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COMPARATIVE SILK PROTEIN EXPRESSION OF DIFFERENT HYBRID VARIETIES OF BOMBYX MORI L.

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ABSTRACT
The present study was undertaken to evaluate production of silk proteins in Kashmiri hybrid varieties of Bombyx mori L. SKUA-R-1×SK28 and SK28 × SK30 against commercially available NB4D2 × SH6 and Mysore evolved CSR2 × CSR4 hybrids. In all the four hybrids, it was observed that the total silk proteins, fibroin and sericin concentrations were more in middle silk gland than in posterior silk gland. This is due to the fact that silk proteins are secreted in the posterior silk gland but stored in the middle silk gland till spinning. The protein concentration in the middle silk gland was highest in Mysore evolved Bombyx mori hybrids CSR2 × CSR4 and commercially used NB4D2 × SH6 followed by Kashmir evolved hybrids SKUA-R-1 × SK28 and SK28 × SK30. The fibroin concentration was observed more in the cocoons of CSR2 × CSR4 and NB4D2 × SH6 followed by SKUA-R-1 × SK28 and SK28 × SK30. The percentage of fibroin was more in CSR2 × CSR4 followed by SK28 × SK30 , SKUA-R-1 × SK28 and NB4D2 × SH6. The sericin concentration was more in NB4D2 × SH6 as compared to other three hybrids. The molecular weight of fibroin and sericin proteins from the silk glands and the cocoons of these four Bombyx mori hybrids were same. The heavy chain fibroin proteins were 350 kDa and the light chain were 25 kDa. The sericin proteins were 66 kDa.

KEY WORDS: Bombyx mori, cocoons, fibrion, sericin, silk glands.

INTRODUCTION

The domesticated silkworm, *Bombyx mori* Linn. a lepidopteran molecular model and an important economic insect that are emerging as an ideal molecular genetic resource for solving a broad range of biological problems (Mondal *et al.*, 2007). The silkworm, *B. mori* produces massive amount of silk proteins during the final stage of larval development. These proteins are stored in the middle silk gland and they are discharged through the anterior duct and spinneret, at the end of the fifth instar (Shimizu, 2000). Two kinds of silk proteins have been distinguished as major components of silk cocoons, the first being fibroin, a fibrous protein composed of heavy (H) chain, Light (L) chain and glycoprotein linked by disulfide bonds Silk fibroin secreted in the lumen of posterior silk gland (PSG) of B. mori consists of three protein component: High (H)-chain 350 k Da (Zhou *et al.*, 2000), Low (L) - chain 26 k Da (Yamaguchi et al., 1989), and Glycoprotein P25 30 k Da (Chevillard *et al.*, 1986). These three types of fibroin (H-chain, L-chain and P 25) are common among different silk producing insects in Lepidoptera, although the fibroin of Saturnidae species secreted as dimer of H-chain and the second being sericin a natural macromolecular protein, serving as an adhesive to unite fibroin for making silk cocoons of silkworm, B. mori.

The sericin of cocoon shell is usually divided into two proportions: (1) a sericin and (2) b-sericin. a- Sericin is present in the outer layer of cocoon shell and b-sericin in the inner layer. The a-sericin contains lesser C and H and somewhat more N and O than the b-sericin (Bose et al., 1989). The solubility of a-sericin in the boiling water is more than b-sericin. Sadov et al., (1987) have come to the conclusion that native sericin is mixture of two substances, sericin A and Sericin B. Recently; silkworm is being used as biofactory for the production of useful protein using the silk gland, which has promoted the technological development in sericulture. With the above background silkworm can be classified as a value added biomaterial for medical application, application of silk protein fibroin and sericin as a biomaterial and other seri-byproducts. Silk fiber is produced by the larvae of certain species of insects for fabrication of cocoons. These cocoons are reeled to produce commercial silk, the primary utilization of silk thread is that it is woven into fine and elegant fabric.

In addition to production of silk fabric, silk is also used in manufacturing racing car tyre, insulation coils for telephones and wireless receivers, sieves for flour mills, fishing lines, parachute coral, surgical stitches etc. Silk is also used as a food additive and in cosmetics. It is also used as support material for enzyme immobilization, contact lens, artificial skin, artificial blood vessels and polymer processing (Iizuka, 2000). Predominantly a proteomic matrix, silk is synthesized by the silk gland cells of silkworm, *Bombyx mori L.* and stored in the lumen of silk glands. Subsequently, it is converted into silk fibers. Each strand of silk fiber is a double structure with two fibroin filaments called brins covered by sericin. The silk fiber is almost a pure protein fiber composed of two types of proteins, namely, fibroin and sericin. It also contains small quantities of carbohydrates, wax and inorganic components which also play a significant role as structural elements during the formation of silk fibers. When the liquid silk is secreted by the silkworms for

spinning, it passes through the anterior gland and is expelled out through the spinneret. It gets stretched by the movement of the larva and solidifies into a stable fiber (Shimizu, 2000). Fibroin, the main component of the silk (70-76%) is exclusively synthesized in the posterior part of the silk gland. It is transferred by the peristalsis into the middle silk gland where it is covered with sericin and is stored until spinning. Fibroin forms the core of the silk thread. It is the fibroin filament which is reeled and woven into silk fabric. Fibroin is composed of eighteen amino acids. Out of these eighteen amino acids, glycine, alanine, tyrosine and serine are the chief constituents and constitutes about 80 per cent of fibroin. Characteristic features of the fibroin in general, are the high proportion of the smaller side group amino acids: glycine, alanine, and serine and their insolubility in aqueous solutions. Sericin is a fine, horny, translucent, yellowish fiber. It is synthesized in different parts of middle division of silk gland. It belongs to a family of proteins having high content of hydroxyl amino acids. The high polarity differentiates the sericin from the fibroin. Sericin is composed of serine (30%), aspartic acid and glutamic acid. It is readily soluble in hot water and in dilute alkali solution. It gets dissolved during the process of boiling of cocoons.

MATERIALS AND METHODS

Silkworms

The experimental material comprised four silkworm hybrids of varied genetic origin viz., $CSR_2 \times CSR_4$, SKUA-R-1 × SK_{28}, $SK_{28} \times SK_{30}$ and $NB_4D_2 \times SH_6$ were reared at the Division of Sericulture, Mirgund under CRD as per the methods suggested by Krishnaswamy (1978) and Dar and Singh (1998).

Isolation of silk proteins from the silk glands and cocoons:

Sericin

Silk proteins were isolated from the silk glands and cocoons by the method described by Rajan *et al.* (1992). Briefly 1-g of silk gland or 1-g of cocoons from each from each hybrid was suspended in 50 ml 0.025 N NaOH solution and stirred for 12 hours at 4 ^{0}C. The soluble protein was extensively dialyzed against double distilled water, concentrated under vacuum and stored at -20^{o}C until use. Alternatively, sericin was extracted by boiling the cocoon fibers in 0.025 percent Na_2CO_3 solution for 1 hour followed by dialysis and concentration, as described by Tashiro *et al.* (1968).

Fibroin

The fibroin was suspended following the method of Yamaguchi *et al.* (1989). Briefly posterior silk gland and shredded cocoon fiber was discriminated by the method described by Rajan *et al.* (1992), dissolved in 60 per cent lithium thiocyanate (LiSCN) solution and incubated overnight at 4^{o}C with gentle stirring. The soluble protein was collected and dialyzed against Tris-HCl buffer (Tris 20 m M, pH 8.0). After sericin was dissolved in 0.025 N NaOH, the left-over silk fiber was treated with 0.1 N NaOH solutions for 24 hours at 4^{o}C with constant stirring. The solution was extensively dialyzed against double distilled water for 2-3 days. The dialyzed sample was centrifuged at 8000 rpm; the supernatant was concentrated and stored at -20^{o}C in aliquots.

Sodium Dodecylsulphate-polyacrylamide gel electrophoresis

Fibroin and sericin isolated from the silkglands(posterior and middle) of each of the hybrids were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmuli *et al.* (1970) in 10% resolving gel (25mA).

Protein estimation

The protein estimation was done as per the method given by Lowry *et al.* (1951). The concentration of the proteins was directly proportional to the formation of blue colored complex read at 750 nm against BSA as standard.

RESULTS

Differential expression of silk proteins in hybrids of *Bombyx mori L.*

The total proteins from the posterior and the middle silk gland were extracted and resolved on the SDS-PAGE for identification of fibroin and sericin. The heavy chain fibroin proteins from these varieties were 350 kDa and the light chain fibroin proteins were 25 kDa on the gel. The sericin proteins were observed at 66 kDa on the gel when compared against the known protein marker (M). There was no change in the molecular weight of these proteins but the intensity of bands varied among different hybrids. Similarly, fibroin heavy chain and light chain were identified on the SDS-PAGE and the molecular weights were same but the expression differed in these varieties. A known protein marker was used as control for comparing the silk proteins and the intensity of the bands was observed as per our estimated protein concentration (Figure 2).

The estimated protein concentration in $NB_4D_2 \times SH_6$ from the posterior and the middle silk gland was 29.2 µg/ml and 48.3 µg/ml, respectively. The estimated protein concentration in the posterior silk gland in this hybrid was more as compared to other three hybrids. The total protein concentration from the posterior and the middle silk gland of $CSR_2 \times CSR_4$ was 24.5 and 52.3 µg/ml, respectively. The concentration of protein in the middle silk gland was higher in $CSR_2 \times CSR_4$ as compared to other hybrids. In SKUA-R-1 × SK_{28}, the total protein concentration in the posterior and in the middle silk gland was 19.5 and 45.6 µg/ml, respectively. Similarly, in case of $SK_{28} \times SK_{30}$ the total protein

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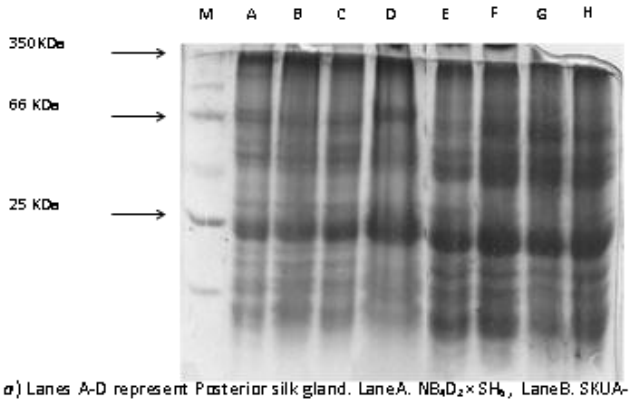
concentration observed in the posterior silk gland was 25 µg/ml and in the middle silk gland it was 43.2 µg/ml. The concentration of the proteins was estimated against the control with Bovine Serum Albumin (BSA) and the standard curve was made accordingly against each silkworm hybrid.

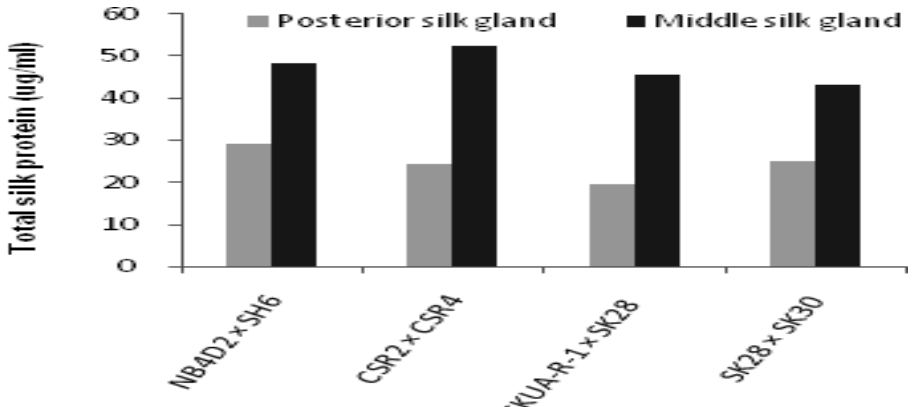
Table 1. : Estimation of proteins from silk glands and cocoons.

	Silk glands				Cocoons			
	$NB_4D_2 \times SH_6$	$CSR_2 \times CSR_4$	SKUA-R-1 × SK_{28}	$SK_{28} \times SK_{30}$	$NB_4D_2 \times SH_6$	$CSR_2 \times CSR_4$	SKUA-R-1 × SK_{28}	$SK_{28} \times SK_{30}$
Total Silk proteins	48.3 (29.2)	52.3 (24.5)	45.6 (19.5)	43.2 (25)	23.5	24.3	23.8	22.9
Fibroin	38.5	43.2	31.8	41.3	18.7	21.3	16.7	16.6
Sericin	29.4	32.1	24.2	23.3	12.3	9.8	9.8	9.6



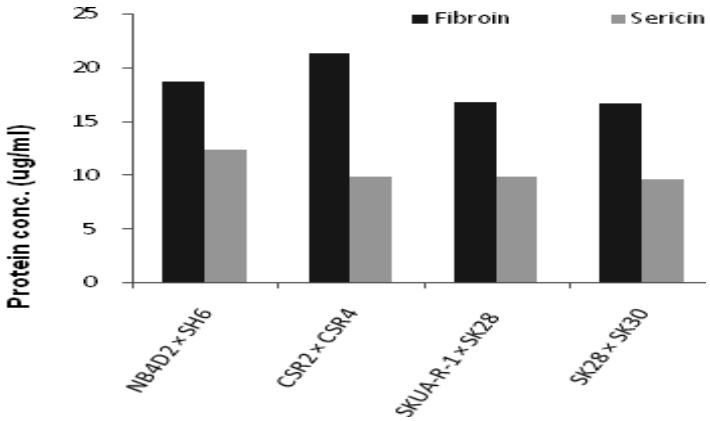
a) Lanes A-D represent Posterior silk gland. LaneA. $NB_4D_2 \times SH_6$, LaneB. SKUA-R-1 × SK_{28}, LaneC. $SK_{28} \times SK_{30}$, LaneD. $CSR_2 \times CSR_4$ *b)* Lanes E-H represent Middle silk gland Lane. E. $NB_4D_2 \times SH_6$, LaneF. SKUA-R-1 × SK_{28}, LaneG. $SK_{28} \times SK_{30}$, LaneH. $CSR_2 \times CSR_4$.

Figure 1. SDS-PAGE gel showing fibroin and sericin proteins from four silkworm hybrids



Comparison of silk proteins produced in four hybrid varieties of silkworms: The proteins were taken from middle and posterior silk glands and estimation was done as described by Lowry *et al.(1951).*

Figure 2a. Estimation of fibroin and sericin proteins from the cocoons and silk glands of *Bombyx mori L.*



Proteins are estimated by methods as described by Lowry *et al* (1951).

Figure 2b. Fibroin and sericin content of cocoons from different hybrids of Bombyx mori L

The concentration of fibroin protein, extracted from cocoons of the hybrid variety of $CSR_2 \times CSR_4$ was 21.3µg/ml and the concentration of extracted sericin protein in this hybrid was 9.8µg/ml. Similarly, the concentration of fibroin protein, extracted from cocoons of SKUA-R-1 × SK_{28} and SK_{28}× SK_{30}, was 16.7 and 16.6 µg/ml, respectively. The concentration of extracted sericin protein of these two hybrids was 9.8 µg/ ml and 9.6µg/ml respectively.

Silk spinning insects mainly belong to two families, viz., Saturniidae and Bombycidae. The mulberry silkworm, *Bombyx mori L.* belongs to Bombycidae. It produces a delicate twin thread of silk fibroin, which is coated by a protective cover of sericin. Silk protein, a kind of structural protein like collagen, elastin and keratin, is an essential constituent of cocoon filament (Komatsu, 1975). Silk fibroin produced by the silkworm, is composed of one heavy (H) ~ chain of ~350 kDa and one light (L) chain of ~25 kDa, which are connected by disulfide bond(s). The H-chain is a fibrous protein and characteristically rich in glycine, alanine, and serine. On the other hand, the L-chain is nonfibrous and contains relatively high amounts of leucine, isoleucine, valine, and acidic amino acids. In the present work, the molecular weight of the fibroin and sericin proteins we obtain from the four silkworm hybrids viz., $NB_4D_2 \times SH_6$, $CSR_2 \times CSR_4$, SKUA-R-1 × SK_{28} and $SK_{28} \times SK_{30}$ are quite similar with the results observed by molecular biologists earlier. However, the estimation of these proteins was different for the silkworm hybrids evaluated in the present study. The estimation of protein concentration was done by the method given by Lowry *et al.* (1951). The concentration of the proteins was estimated against the control with Bovine Serum Albumin and the standard curve was made accordingly against each hybrid variety.

The present study has given a status of estimation of the total protein concentration of these four hybrid varieties. The maximum concentration of proteins in posterior and the middle silk gland of $NB_4D_2 \times SH_6$ were 29.2 and 48.3 µg/ml, respectively. This hybrid had maximum concentration in the posterior silk gland as compared to other three hybrids. The total protein concentration in posterior silk gland and in the middle silk gland of $CSR_2 \times CSR_4$ was 24.5 and 52.3 µg/ml, respectively. The concentration of protein in the middle silk gland was higher in $CSR_2 \times CSR_4$ as compared to other hybrids. SKUA-R-1 × SK_{28} had a protein concentration of 19.5µg/ml in the posterior silk gland and 45.6 µg/ml in th middle silk gland . Similarly, in case of $SK_{28} \times SK_{30}$ the protein concentration observed in the posterior silk gland was 25 µg/ml and in middle silk gland it was 43.2 µg/ml. In all hybrids, protein concentration was lower in posterior silk gland as compared to middle silk gland.

This is because the proteins move from posterior silk gland to middle silk gland due to peristaltic movements in the mature silkworm larvae and therefore maximum protein accumulation takes place in the middle silk gland. The middle silk gland stores the silk proteins. The fibroin protein concentration, extracted from cocoons of the hybrid variety of $CSR_2 \times CSR_4$ hybrid cocoons was 21.3 µg/ml and the extracted sericin protein of the same variety was 9.8 µg/ml. When the concentration of sericin in cocoon was compared with total protein concentration in the middle silk gland of $CSR_2 \times CSR_4$, it confirmed the results that sericin was less in this hybrid. Similarly fibroin protein concentration, extracted from cocoons of the hybrids SKUA-R-1 × SK_{28} and $SK_{28} \times SK_{30}$, was 16.7 and 16.6 µg/ml and the concentration of extracted sericin protein from these varieties was 9.8 and 9.6 µg/ml respectively. The proportion of fibroin and sericin proteins in four hybrids shows that $CSR_2 \times CSR_4$ has got highest percentage of fibroin followed by

SK_{28} × SK_{30} and SKUA-R-1 × SK_{28}. More the fibroin content better is the silk recovery percentage. However, other genetic attributes like viability etc. are also to be taken into consideration for selecting a silkworm hybrid for its commercial exploitation.

In the present study no change in the molecular weight of these proteins was observed when compared to previous data, except that the intensity of bands were high and that may be because of variation in the protein concentrations (Fig1). Similarly, fibroin heavy chain and light chain were identified on the SDS-PAGE and the molecular weights were same but the expression differed in these varieties. The sericin protein came on 66 kDa when compared against the known protein marker. Similarly fibroin and sericin protein were observed as per their molecular weight i.e., fibroin heavy chain at 350 kDa, light chain at 25 kDa and sericin at 66 kDa in all the varieties. Known protein marker was used as control for comparing the silk protein. Again the intensity of the bands was observed as per our estimated protein concentration (Figure 2b.)

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