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Biochemical Analysis of Haemolymph of *Antheraea mylitta*

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Abstract

The objectives of this study on the "*Biochemical analysis of haemolymph of Tasar silkworms*" is to search base line ways and means for improving silk performance attributes which are needed in generating rural livelihood employment and earning of foreign revenues. *Antheraea mylitta* Drury is a holometabolous insect with eggs, larva, pupa, cocoon and adult are stages in its life cycle. There are five larval instars which feed on the primary host plants like Arjun (*Terminalia arjuna*), Asan (*Terminaliatomentosa*), Sal (*Shorearobusta*) apart with a number of host plants of secondary importance. The larvae voraciously feed on the leaves of their host plants and 5th instars larva spins the silk around it and forms the cocoon from which Tasar silk is reeled. Silkworms are being used as bio-factory for the production of useful silk proteins which are natural polymers and are biodegradable, with reactive functional groups that open possibilities to link the crops with other polymers to be used in controlled delivery system.

Keywords: Haemolymph; Tasar silk; SDS; Trehaelose; Protein; Larvae; Silkworm

Introduction

Silk is the traditional agriculture product and the Asian region is the primary supplier of silk in the world market. Among all silk producing countries, China account for the bulk of silk export to the world. However, silk producing countries of the Asian region are now experiencing significant change in their production patterns. The countries of the region with advanced sericulture, such as *JAPAN* and *REPUBLIC OF KOREA*, have experienced an apparent irreversible decline in their silk output level. At the same time, other countries have realized sericulture's potential benefits such as enhancing foreign exchange earnings as well as alleviation of rural poverty through employment and income generating activities. Many developing countries of the region, therefore are undertaking intensive effort to rehabilitate their silk industries, while some have recently upon sericulture for the first time.

Like other common biomedical textiles such as polyester, silk containing various polar functional groups that might enhance antibiotic absorption. Silk is eco-friendly, biodegradable polymers with excellent moisture-absorbing and deabsorbing properties. Sericin and fibroin the two proteins collectively make the silk fibre, which in turn made from soluble protein present in the haemolymph. Haemolymph is the store house of many biochemicals constituent which serve as raw materials for the synthesis of sericin and fibroin. Sericin and fibroin have been recently explored in the field of drug delivery system.

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Fibroin has been used in a number of biomedical applications and has good wound healing properties. Sericin coated powders are used as cosmetics for dermatitis inhibitor as wound protection film, nail cosmetics and chewing gums. Silk protein is a biomaterial with anticoagulant properties. The fibroin protein is a kind of biological material used for artificial skin and in other medical applications. Thus, silk proteins secreted by Tasar silkworms are used in biomedical applications since very long period by mankind. Thus, Tasar silk has great role in textile industry, environmental conservation, wild life management, biomedical applications, pharmaceuticals and earning foreign exchange revenues. Considering such significances of silkworms, the present study on the **"*Biochemical analysis of haemolymph of Tasar Silkworms*"** has been undertaken. Silk is a natural protein fibre, some forms of which can be woven into textiles. The protein fibre of silk is composed mainly of fibroin and produced by certain insect larvae to form cocoons. The best-known type of silk is obtained from the cocoons of the larvae of the mulberry silkworm *bombyx mori* reared in captivity domestication. The shimmering appearance of silk is due to the triangular prism-like structure of the silk fibre, which allows silk cloth to refract incoming light at different angles, thus, producing different colours. Silks are produced by several other insects, but generally only the silk of moth caterpillars has been used for textile manufacturing. There has been some research into other silks, which differ at the molecular level. Silk is lower in density than cotton, wool or nylon and as such, is highly moisture absorbent, able to absorb as much as a third of its own weight the moisture without feeling damp.

Silk Production

Silk is a continuous-filament fiber consisting of fibroin protein, secreted from two salivary glands in the head of each larva, and a gum called sericin, which cements the two filaments together. The sericin is removed by placing the cocoons in hot water, which frees the silk filaments and readies them for reeling.

Properties of Silk

Silk is one of the popular fabrics for apparel because of its unique properties. Silk is most luxurious fabric, the most comfortable fabric, the most absorbent of fabrics (equal to wool), the best fabric for drape, the best fabric for color, capable of the greatest luster, having the finest "hand" etc. These are some of the factors which make the fabric more popular. The fabric is cool in summer and warm in winter.

Physical properties

The silk fiber is chiefly composed of 80% of fibroin, which is protein in nature and 20% of sericin, which is otherwise called as silk gum. Silk as a fiber, has good tensile strength, which allows it to withstand great pulling pressure. Silk is the strongest natural fiber and has moderate abrasion resistance. The strength of the thrown yarns is mainly due to the continuous length of the fiber. Spun silk yarn though strong is weaker than thrown silk filament yarns. Silk fiber is an elastic fiber and may be stretched from 1/7 to 1/5 of its original length before breaking. It tends to return to its original size but gradually loses little of its elasticity. This would mean that the fabric would be less sagging and less binding resulting in the wearers comfort. Silk has a liability and suppleness that, aided by its elasticity and resilience, gives it excellent durability. Silk is a protein fiber and is a non-conductor of heat similar to that of wool. This makes silk suitable for winter apparel. Silk fabrics being protein in nature have good absorbency. The absorptive capacity of the silk fabric makes comfortable apparel even for warmer atmosphere. Fabrics made from silk are comfortable in the summer and warm in the winter. Silk fiber can generally absorb about 11 percent of its weight in moisture, but the range varies from 10 percent to as much as 30 percent. This property is

also a major factor in silk's ability to be printed and dyed easily. Silk fabric does not attract dirt because of its smooth surface. The dirt, which gathers can be easily removed by washing or dry cleaning. It is often recommended for the silk garments to be dry-cleaned. Silk fabrics should always be washed with a mild soap and strong agitation in washing machine should be avoided. Silk water – spot easily, but subsequent washing or dry cleaning will restore the appearance of the fabric. Silk fabrics are subjected only to normal shrinkage which can be restored by ironing. Crepe effect fabrics shrink considerably in washing, but careful ironing with a moderately hot iron will restore the fabric to its original size. Silk is sensitive to heat and begins to decompose at 330°F (165°C). The silk fabrics thus have to be ironed when damp. Silk fabric weakens on exposure to sun light. Raw silks are more resistant to light than degummed silk. Silks will not mildew unless left for some time in a damp state or under the extreme conditions of tropical dampness. Silk may be attacked by the larvae or clothe moths or carpet beetles. Perspiration and sunlight weakens and yellows silk fabrics. The silk itself deteriorates and the color is affected causing staining. Garments worn next to the skin should be washed or otherwise cleaned after each wearing.

Chemical properties

Silk, like wool, is deteriorated with chlorine bleaches like sodium hypochlorite. However, mild bleach of hydrogen peroxide or sodium per borate may be used for silk. Silk is not as sensitive as wool to alkalis, but it can be damaged if the concentration and the temperature are high. A mild soap or detergent in lukewarm water is thus advisable. Concentrated mineral acids will dissolve silk faster than wool. Organic acids do not harm silk. Silk has good absorbency and thus has good affinity for dyes. Dyed silk is color fast under most conditions, but its resistance to light is unsatisfactory.

Principal Proteins Present in Silk

Fibroin

The fibroin protein consists of layers of antiparallel beta sheets. Its primary structure mainly consists of the recurrent amino acids sequence (Gly-Ser-Gly-Ala-Gly-Ala)$_n$. The high glycine (and to a lesser extent, alanine) content allows for tight packing of the sheets, which contributes to silk's rigid structure that can't be stretched (tensile strength). A combination of stiffness and toughness make it a material with applications in several areas, including biomedicine and textile manufacture. Fibroin is known to arrange itself in three structures, called silk I, II, and III. Silk I is the natural form of fibroin, as emitted from the *Bombyx mori* silk glands. Silk II refers to the arrangement of fibroin molecules in spun silk, which has greater strength and is often used in various commercial applications. Silk III is a newly discovered structure of fibroin. Silk III is formed principally in solutions of fibroin at an interface (i.e. air-water interface, water-oil interface, etc.).

Sericin

Sericin is refined from silkworm body (spun silk gland), pod (pod shell, watt silk) and silk. There are 18 kinds of amino acids in sericin among which serine and aspartate have the highest content. Besides, it has other amino acids necessary to human body. It is an excellent protein. Because in sericin, about 80% amino acids have hydrophilic lateral group, about 1/3 of which is serine in which water absorption is 50 times high than that of glycerine. Sericin, as the raw material of cosmetic, has excellent moisture absorption and preservative ability. Sericin protein can form a film on the surface of skin and hair so that the water in skin can be preserved, and then harm to skin cutin can be avoided. Sericin is excellent in moisture preservation as it contains serine, one of the most important amino acids among the NMF. It is also effective in controlling the occurrence of active oxygen, the factor which breaks down the protection of the skin's surface, as well as tyrosinase, which causes the

skin to freckle. As it is gentle on the skin and is an excellent moisturizer, protector and whitening, it is attracting attention as a skin care ingredient.

Review of Literature

The study on the biochemical studies of haemolymph of tropical silkworm *Antheraea mylitta* are investigated [1]. The morphological changes, structure and function of silk gland were studied and reported [2,3]. The ultra-structure of the posterior silk gland cells and liquid silks in Indian Tasar silkworms *Antheraea mylitta* Drury have been reported [4-6]. On studying the biochemical, physiological and electrophoretic mobility function of haemolymph in Tasar silkworms the developmental stage, structure and secretary activity of the tropical silkworm are noted [7-10]. Various biochemical studies revealed periodic changes in the total concentration of haemolymph amino acids and protein during metamorphosis in insects [11-15]. Trehalose is the major and metabolically active, non-reducing disaccharide in the insect blood [16-21]. A well-established report says that insect blood or haemolymph is a store house of various kinds of proteins and enzymes [22-25]. Owing to economic and commercial significance, silk output has stimulated workers to investigate the silk production mechanism with reference to silk gland [26-28]. In India, various aspects including the techniques of culture of non-mulberry silks were studied and classified [29-41]. The Indian subcontinent is the home for non-mulberry silkworms and their rearing because of the extreme fluctuation of climatic conditions. Among non-mulberry silks, Tasar silkworms and its rearing are the most important sericulture activities. The structure and function of silk gland cells and the semi-domestication of tropical Tasar silkworms were real contributions in the field of Tasar sericulture [42,43]. The quantitative and qualitative changes of the soluble protein in the various tissues including haemolymph of the developing stages of the tropical silkworm *A. Mylitta* are also interpreted [44-47]. Some contribution on the quantitative and qualitative changes in protein profiles of various tissues of tropical silkworms, *Antheraea mylitta* were made [48-50]. The changes in protein and total sugar content in eri-silkworm, *Philosomiaricini* during 5th instar development is evaluated. The electrophoretic studies on developmental profiles of proteins in haemolymph, fat body and ovary of the red cotton of *Dysdercus cengulatus* [51-56] are discussed. It is well known that haemolymph the only extra-cellular fluid in insects is having diverse functions [57]. It is the reservoir for most of the biochemical constituent that are required for nearly every physiological activity of the insect. Thus, the change in the composition of the haemolymph reflects the inorphogenic and biochemical changes taking place in insect tissues. The reported on the electrophoretic studies on development profiles of proteins in haemolymph, fat body and ovary of red cotton bug *Dysdercus koenigii are also examined.* Different aspects of silk gland have been nicely reviewed by a number of workers [58]. The insect haemolymph performs several physiological functions such as immunity, transport, storage etc. [59]. Insects need carbohydrates as major fuel for their growth and development being deprived mostly from the diet. The Tasar silkworms *Antheraea mylitta* conserves sufficient quantity of energy reserves in larval stage to be utilized during pupation and adult stages. The pioneer works on the subject has been carried out with the advent of the biotechnology and modern subject like DNA recombinant technology to make hybrids for making silkworms or adaptive to changing environmental conditions. The application of the biotechnological tools and techniques is to produce or silks and also to increase in feeding period of the larval instars. Juvenile hormone analogue (JHA) is known to prolong the larval duration in insects and these have been utilized for the improvement of silk production in the silkworm *Bombyx mori.* The available literature on the "Biochemical analysis of haemolymph of Tasar silkworms" is only available in the first decade of 21st century. On further investigation the soluble proteins content in leaves of different age groups of *T. Arjuna*, the primary host plant of Tasar silkworms are highlighted [60]. The endogenous 20-hydroyeedysome levels in haemolymph of non-dispense diapauses and

diapauses are destined generations of Tasar silkworms, *Antheraea mylitta*. The purification characterization and immunolization of a novel protease inhibitor from haemolymph of Tasar silkworm *Antheraea mylitta* are highlighted to study [61]. The work of similar nature has been carried out by various investigators on Tasar silk worm. The soluble protein content of the leaves of *Terminalia arjuna*, the primary host plant of the Tasar silk worm are analysed and compare with various environmental factors [61]. A series of valuable works on the Chinese Tasar silkworms in relation to environmental factors perhaps the first authentic knowledge on Tasar culture [62-67]. The screening of pertinent literature revealed that the Tasar silkworms rearing technology and its significances have been realized in the beginning of the present century. The literature survey revealed the environmental factors on Tasar silkworms. A complete review of evolution of arthropod silks has been made. By using electrophoretic and antigen-antibody precipitation method a good number and quantity of different fractions of proteins have been identified and these vary depending on the stage of insect development as well as its eco-physiological conditions. The major haemolymph proteins which have been characterized so far include storage proteins, lipoproteins, ovarian proteins, anti-bacterial proteins, lecting protease inhibitors, different enzymes, chromo proteins, metal binding proteins and hormone carrier proteins. One of the most striking features of the development of holo-metabolous insects is the synthesis of a quantitatively significant class of polypeptides known as storage proteins. These proteins comprise the bulk of the polypeptides in larval haemolymph constituting about 80% of the total haemolymph proteins which are characterized by exceptionally high content of aromatic amino acids viz tyrosine and phenyl amine. The insect storage proteins are high molecular weight usually hexamers, compound of sub unity in molecular range of 70 kDa to 90 kDa. The storage proteins appear to be spiral adaptation to insects in molting metamorphosis and reproduction. Fat body and pericardial cells contribute to the haemolymph protein pool. Considerable work has been done on the estimation of soluble protein in insects but literature pertaining to the analysis of haemolymph protein of Tasar silk worm is scanty except the work of fuel biochemist (FIG. 1-8).

Total soluble protein estimation

The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9.0 to 10.5 is essential. The leaves of the host plants are the primary source of proteins in silkworms (TABLES 1-8). The quality and quantity of proteins in these insects depends upon the young, semi-mature and mature leaves of host plants. After digestion, amino acids and proteins present in leaves pass into haemolymph. The haemolymph, the only extracellular fluid in insects has plasma in which haemocytes are suspended. The plasma, having diverse functions such as immunity, transport and which bathes all the tissues that constitute 5% to 40% of the total storage products required for cellular body weight of an insect and contains many organic substances that regulate metabolism. Thus, the changes in the biochemical organic/inorganic constituents in haemolymph control regulatory functions. The composition of haemolymph reflects the morphogenic changes of insects, Almost all inorganic constituents like electrolytes or ions, biochemical changes in the body of the insect in response to phosphates and organic constituents like free amino to ambient environment. High temperature affects nearly acids, proteins, lipids, carbohydrates, uric acid etc. All biological processes including the structure of proteins depend upon food derived from host plants of silkworms. The host plant Arjun/Asan leaves is the source of proteins and other essential nutrients for Tasar silkworms. It is relevant to know that soluble protein present in the haemolymph of different developmental instars of Tasar silk worm for predicting the quality and quantity of silk fibre to be synthesized. The

objective of the present study is to quantify the soluble protein present in the haemolymph of the pupal stage of the *Antheraea mylitta*. Considering such objective, the present study has been undertaken and different concentration of protein present is evaluated. Comparing the biochemical analysis of haemolymph another similar work determining the protein content of the leaves of host plant *Terminalia arjuna*, the primary host plant of Tasar silkworm are also analysed and compare with various environmental factors. After analysing the total protein content, the sugar content (trahelose) present is further investigated and discuss in general. Therefore, different methodology is used to analyse different biological components present in the haemolymph of *Antheraea mylitta.*

Principle: The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein will produce a blue purple colour complex, with maximum absorption in the region of 660 nm wavelength, with Folin-Ciocalteau reagent which consists of sodium tungstate, molybdate and phosphate. Thus, the intensity of colour depends on the amount of these aromatic amino acids present and will thus, vary for different proteins. Most proteins estimation techniques use bovine serum albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive down to about 10 µg/ml and is probably the most widely used protein assay despite its being only a relative method, subject to interference from Tris buffer, EDTA, non-ionic and cationic detergents, carbohydrate.

Methodology

Determining the soluble protein content present in the haemolymph

Haemolymph was collected in a pre-chilled test tube containing a few crystals of thiourea by cutting the first proleg of larva. The haemolymph was conditioned. The haemolymph was centrifuged at 3000 rpm for 10 min at 4°C and the supernatant proteins were used in the protein estimation. The protein estimation was done with the help of Lowry method. Following reagents are required for the total soluble protein estimation:

1. (BSA) solution (1 mg/ml). 2. Analytical reagents: 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solutions. 10 ml of 1.56% $CuSO_4$ mixed with 10 ml of 2.37% Sod. Potassium tartarate solution. Analytical reagents by mixing 2 ml of (solution B) with 100 ml of (solution A). Solution B: 2 gm Na_2CO_3 and 0.4 gm NaOH volume make up with 100 ml distilled water. Solution C: solution A (1 ml.)+B (50 ml). Solution D: 0.25 ml FCR (freshly prepared).

Observation:

TABLE 1. **Preparation of standard curve of protein.**

T.T. No.	Amount of sample (ml)	Amnt of sol. C (ml)	Amnt of FCR (ml)	OD (750 nm) (Mean value)
1	0.0	2.5	0.25	0.000 (Blank)
2	0.1	2.5	0.25	0.146
3	0.2	2.5	0.25	0.206
4	0.3	2.5	0.25	0.250
5	0.4	2.5	0.25	0.316
6	0.5	2.5	0.25	0.386
7	0.6	2.5	0.25	0.438
8	0.7	2.5	0.25	0.478
9	0.8	2.5	0.25	0.523
10	0.9	2.5	0.25	0.597
11	1.0	2.5	0.25	0.672

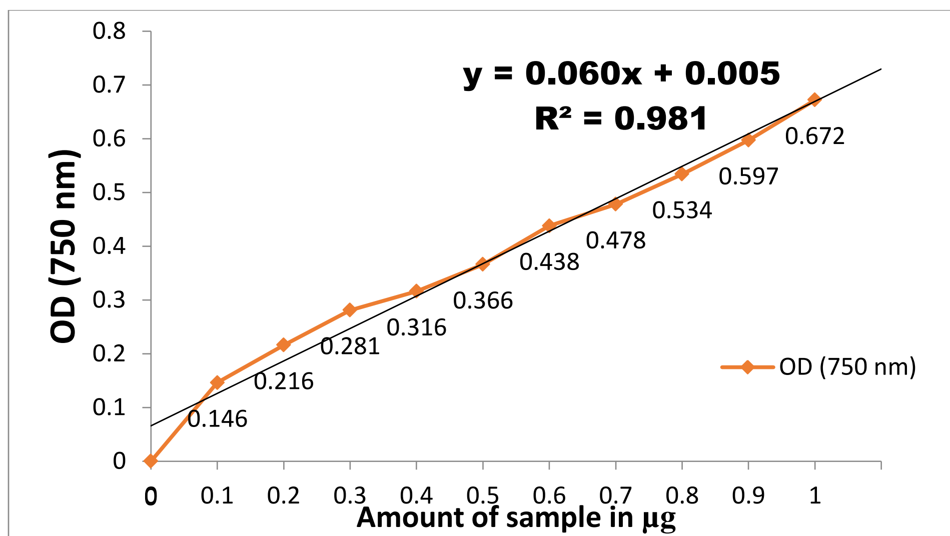

FIG. 1. **Plotting the standard curve of protein by comparing OD vs. amount of sample.**

TABLE 2. **Showing the OD and concentration of protein in haemolymph (for the sample).**

Test tube No.	OD (750 nm)	Conc. (µg)
01	0.310	38.0
02	0.302	37.0
03	0.306	37.5
04	0.314	39.0
05	0.308	37.7
06	0.312	38.5
07	0.310	38.0
08	0.306	37.5
09	0.308	37.7
10	0.312	38.5

Calculation of standard error

$$SE=\frac{\sum (A-\bar{A})^2}{n-1} \quad (1)$$

where, A=Numerical value, A=Mean value, n=total no. of observations.

Determining the soluble protein content of the leaves of *Terminalia arjuna*

These works are in conformity with the present study. The method of electrophoresis is used to determine the soluble protein content of this plant which is a primary host of the Tasar silk worm. Following reagents were used: Acryl amide**,** Bisacrylamideis (N, N' methylmetrisacrylamide)**,** Tris (2-hydroxymethyl-1, 3-propanediol)**,** SDS, TEMED, Ammonium per sulphate, Beta mercaptoethanol, (2-mercaptoethanol)**,** Glycerol, Bromophenolblue, Glycine, Hydrochloric acid (HCl), Dithithreitol (DTT).

Stock solution: (a) 2 M-Tris HCl (pH 8.8), 100 ml (b) 1 M Tris HCl Ph 6.8, 100 ml (c) 10% w/v SDS, 100 ml, store at room temperature (d) 50% w/v Glycerol, 100 ml (e) 1% w/v Bromo phenol blue, 10 ml.

Working solution: (1) Solution A (Acryl amide stock solution, 100 ml) 30% (w/v) and acryl amide 0.8% (w/v) Bisacrylamide. To 29.2 g acryl amide and 0.8 gm bisacrylamide, add distilled water to 100 ml and stir until completely dissolved. Work under hood and keep acryl amide solution covered with parafilm until acryl amide powder is completely dissolved.

(2) Solution B (4x separating gel buffer, 100 ml) 75 ml 2 M Tris HCl (pH 8.8)-1.5 M, 4 ml 10%SDS -0.4%, 21 ml H_2O;

(3) Solution C (4x stacking gel buffer, 100 ml) 50 ml 1 M Tris HCL (pH 6.8))-0.5 M, 4 ml 10%SDS -0.4 M, 46 ml H_2O;

(4) Solution D (10% Ammonium per sulphate, 5 ml) 0.5 gm ammonium per sulphate, 5 ml H_2O;

(5) Solution E (Electrolysis buffer, 1 litre) 3 g Tris, 14.4 g glycine, 1 gm SDS, H_2O to make 1 lit;

(6) 5x Sample buffer, 10 ml (0.6 ml 1 M Tris HCL pH 6.8, 5 ml 50% glycerol, 2 ml 10% SDS, 1 ml 1% Bromophenol Blue, 0.9 ml H_2O.

Gel casting: Water was poured covering the separating gel. After those solutions A, C and water was mixed in a small flask. Ammonium per-sulfate and TEMED were added and mix gently by inverting the container. After that pipette stacking gel solution was transferred onto separating gel until solution reached to top of the front plate. Then carefully insert comb in to gel sandwich until bottom of teeth reach top of front plate. Leave the stacking gel to polymerize. After stacking gel has been polymerized, remove the comb carefully. After that place, the gel into electrophoresis chamber. Then add electrophoresis buffer to inner and outer reservoir, making sure that both top and bottom of gel are immersed in buffer.

Sample preparation: The sample for the electrophoretic molecular weight determination of sericin and fibroin was prepared separately. Sericin is of hydrophilic nature. Thus, their solubility is different in water. Sericin was extracted by dissolving the silk threads into hot water maintained at 80°C in water bath. The sericin extract was brought to the normal temperature and then precipitated with the help of cold TCA at final concentration of 5%. The precipitated sericin was pelleted by centrifugation. The pellets were retained while supernatant was discarded. For the removal of lipids, the pellet were re-suspended by acetone/ethanol solution and centrifuged at low speed. Supernatant containing lipids was again discarded. The pellets were resuspended with phosphate buffer and stored at 4°C for further estimation. Silk threads after sericin removal were used for the extraction of fibroin. The water boiled silk threads were washed for 3 times in hot water (80°C) and used for fibroin extraction. Fibroin, due to hydrophobic in nature, does not dissolve in water. Fibroin was extracted from the silk thread by dissolving in glacial acetic acid (10%). Silk thread was soaked into glacial acetic acid (10%) solution and vortexes for about 5 mins under normal condition. Dissolve fibroin was precipitated with the help of cold TCA (at final conc. of 5%). The precipitated fibroin was pelleted by centrifugation. The pellets were retained while supernatant was discarded. For the removal of lipids, the pellets were resuspended by Acetone/Ethanol solution and centrifuged at low speed. The pellets were resuspended with the phosphate buffer and stored at 4°C for further estimation.

Sample loading: Rinse the syringe to be used for loading samples a few times with distilled water. Insert the syringe to about 1 mm to 2 mm from the well bottom before delivery. Rinse the syringe a few times with distilled water after loading. Load the second and other well with 2:l of samples. Do not pipette the pellet at the bottom of the microfuge tube. Rinse the syringe a few times with distilled water after loading.

Running the gel: Check that the buffer in the upper buffer chamber is full because leakage of the buffer may occur. Place the lid on top of the lower buffer chamber. Make sure that the connection is correct. Attach the electrical leads to a suitable power pack with the proper polarity. Run the gel at a constant current of 30 mA. Stop the electrophoresis when the tracker dye is ~ 1 cm above the end of the glass plates.

Removing and staining of the gel: Gel from the buffer chamber was removed. Loosen all four screws of the clamp assembly and remove the glass plate sandwich from it. Push one of the spacers out to the side of the plates without removing it. Gently twist the spacer so that the upper glass plate pulls away from the gel. Cut the gel on one side (to orientate the gel). Remove the gel by gently grasping two corners of the gel and place it in the container containing the Coomassie blue stain. Make sure that the gel is fully submerged in the staining solution. Stain the gel for 1 h; agitate it slowly on a shaker. Destain

the gel in a destaining solution a few times until protein bands are visualized. Approximately determine the molecular weight of the visualized protein bands by comparing them with the molecular weight markers.

TABLE 3. **Molecular weight of the unknown protein.**

Proteins	Mr ($\times 10^{-3}$)
Myosin	205
β-galactosidase	116
Phosphorylase b	97
Albumin	66
Glutamate dehydrogense	55
Ovalbumin	45

Calibration curve can be generated in several forms.

a. Relative molecular weight vs. migration distance.

b. Log relative molecular weight vs. migration distance**.**

Graphs obtained by plotting molecular weight vs. migration distance yield a hyperbolic curve, making molecular weight determination difficult. An almost linear relationship is, however, obtained when logarithm (molecular weight) is plotted vs. migration distance. The molecular weight of the unknown proteins can be determined from the regression or directly from the graph. Plotting logarithm (molecular weight) vs. relative migration distance (Rf) is required for comparing the results obtain from separate gel.

$$R_f = \frac{Calibrator\ migration\ distance\ (mm)}{Bromophenol\ blue\ migration\ distance\ (mm)} \quad (2)$$

FIG. 2. **Relative molecular weight vs. migration distance.**

FIG. 3. **Graph showing the log of relative molecular weight vs. migration distance.**

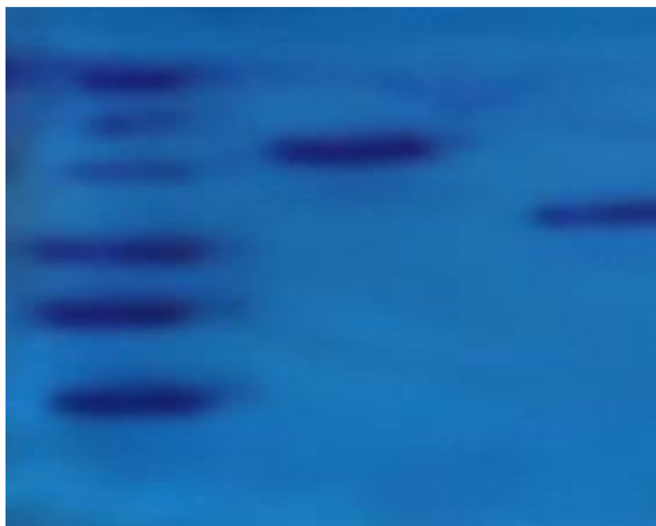

FIG. 4. **SDS bands after destaining.**

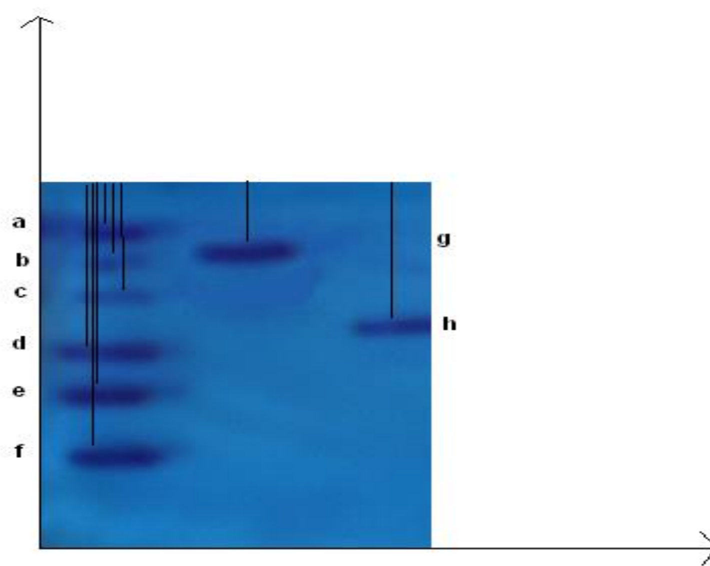

FIG. 5. **Graphical representation of the bands.**

TABLE 4. **Showing relative front (mm) of standard protein and sample protein.**

Standard protein	Distance moved by standard protein
A	3
B	6
C	8
D	12.
E	14
F	17

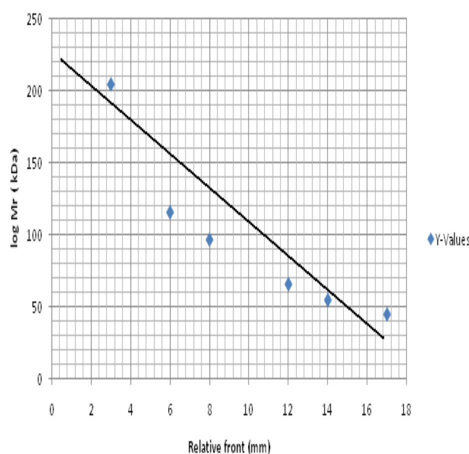

Fig 6. **Comparison of known protein with standard curve.**

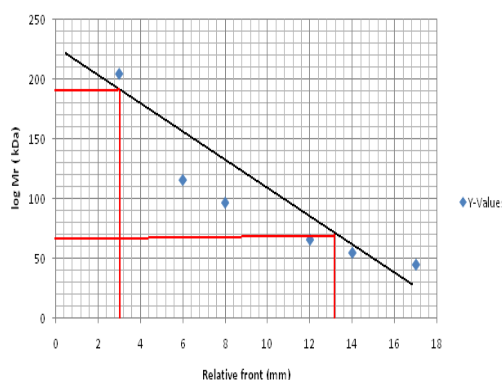

TABLE 5. **Showing the results of the relative molecular weight of the sample proteins.**

Distance moved by sample protein (mm)	**Molecular weight of sample protein (kDa)**
3.1	195-198
12.2	65-70

Determining the sugar content by spectrophotometric estimation by Anthrone method

The haemolymph works as a circulatory fluid, which supplies all the nutrients and permit gaseous exchange to the tissues. The presence of different types of sugars in the haemolymph can be estimated through Anthrone or Phenol method. These methods are reliable and used extensively for the quantitative estimation of sugar present in the sample. The most valuable sugar present in the haemolymph is the trehalose (α, α-1, D Glucopyranose), a dimmer of two glucose units. This trehalose cannot be estimated as such, but through designing the experiment for trehalose estimation, it is possible to estimate total sugar content and the amount of trehalose present in the sample at a particular Standing state. Trehalose can be digested into its monomers (Glucose) by an enzyme known as trehalase, this enzyme specifically digest the trehalose into glucose and make it possible to estimate both total sugar content and trehalose content of the sample. The sugar sample containing

trehalose gets digested into glucose monomers which react with anthrone and phenols to form a coloured compound. Thus, the sample can be estimated for total sugar. This method can also be used for the estimation of reducing as well as non-reducing sugars and total sugar in the sample. In this method, the sugars in presence of concentrated sulphuric acid get dehydrated and produce furfural (from pentose) or 5-hydroxy methyl furfural (5-HMF) (from Hexose), which when reacted with anthrone produces a coloured compound with λ_{max} of 620 nm. Pentose, hexoses, heptoses and their derivatives yield a coloured product in these reactions whereas trioses, tetroses and amino sugars do not yield any coloured product. Anthrone method is simple, insensitive to interference and therefore, gives a reliable index of total carbohydrate in the sample.

Reagents: Trehalase (α, α-Trehaloseglucohydrolase), anthrone reagent (0.2%, freshly prepared) dissolve 200 mg anthrone in 5.0 ml distilled ethanol and make up to 100 ml with 75% sulphuric acid and standard sugar solution (0.1%) dissolve 100 mg of trehalose in 100 ml distilled water. Dilute 1:10 for estimation.

TABLE 6. **Number of reagents for Anthrone method of estimation.**

Test Tube No.	Sample (ml)	D.W (ml)	Trehalase (µl)	Anthrone reagt (ml)	Conc. sulphuric acid (ml)	O.D (λ620 nm)
1	0.0	1	1.0	1.0	5.0	0.00
2	0.1	0.9	1.0	1.0	5.0	0.122
3	0.2	0.8	1.0	1.0	5.0	0.192
4	0.3	0.7	1.0	1.0	5.0	0.249
5	0.4	0.6	1.0	1.0	5.0	0.379
6	0.5	0.5	1.0	1.0	5.0	0.462
7	0.6	0.4	1.0	1.0	5.0	0.538
8	0.7	0.3	1.0	1.0	5.0	0.636
9	0.8	0.2	1.0	1.0	5.0	0.751
10	0.9	0.1	1.0	1.0	5.0	0.835
11	1.0	0	1.0	1.0	5.0	0.928

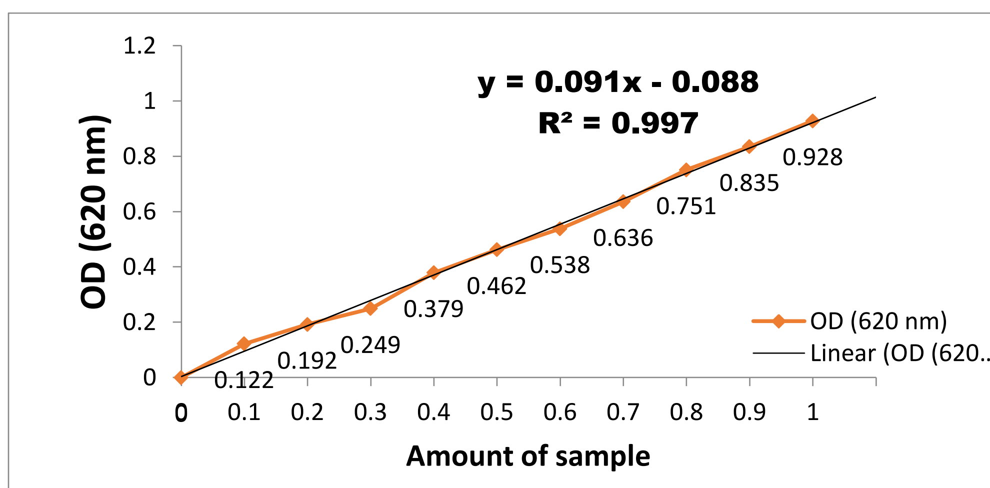

FIG. 7. **Standard curve for total sugar content.**

TABLE 7. **Tabulation showing the total amount of sugar content.**

Test tube no.	OD (620 nm)	Conc. (µg)
01	0.42	0.47
02	0.46	0.49
03	0.38	0.43
04	0.39	0.44
05	0.41	0.46
06	0.42	0.45
07	0.43	0.46
08	0.41	0.45
09	0.41	0.46
10	0.39	0.42

From the table, we found the estimated value of total sugar varied from 0.4523 µg to 0.4477 µg.

Trehalose estimation by spectrophotometric phenol method

The haemolymph works as a circulatory fluid, which supplies all the nutrients and permits nutrient exchange to the tissues. The presence of different types of sugars in the haemolymph can be estimated through Anthrone or Phenol method. These methods are reliable and used extensively for the quantitative estimation of sugar present in the sample. The most valuable sugar present in the haemolymph is the trehalose, a dimmer of two glucose units. This trehalose cannot be estimated as such, but through designing the experiment for trehalose estimation, it is possible to estimate total sugar content and the amount of trehalose present in the sample at a particular standing state. Trehalose can be digested into its monomers (glucose) by an enzyme known as trehalose, this enzyme specifically digest the trehalose into glucose and make it possible to estimate both Total Sugar content and trehalose content of the sample. The sugar sample containing trehalose gets digested into glucose monomers which react with anthrone and phenols to form a coloured compound. Thus, the sample can be estimated for total sugar. The concentration of trehalose can be estimated by deducing the concentration of total sugar of the sample digested with Trehalase to the concentration of undigested sample.

Conc. of trehalose=(Conc. of total sugar of the sample after digestion – conc. of sugar of undigested sample).

This method can also be used for the estimation of reducing as well as non-reducing sugars and total sugar in the sample. In this method, the sugars in presence of concentrated sulphuric acid get dehydrated and produce furfural (from pentose) or 5-hydroxy methylfurfural (5-HMF) (from Hexoses), which when reacted with phenol produces a coloured compound with λ_{max} of 490 nm. Pentose, hexoses, heptoses and their derivatives yield a coloured product in these reactions whereas trios, tetras and amino-sugars do not yield any coloured product. A phenol method is simple, insensitive to interference and therefore, gives a reliable index of total carbohydrate in the sample.

Reagents used: Trehalose (α, α-trehalose glucohydrolase), phenol reagent (5.0%, freshly prepared) dissolve 5 g Phenol in 100.0 ml distilled water. Standard sugar solutions (0.1%) dissolve 100 mg of trehalose in 100 ml distilled water. Dilute 1:10 for estimation.

Observations:

TABLE 8. **The number of reagents for estimation of trehalose by phenol method.**

Test tube no.	Sample (ml)	D.W (ml)	Trehalase (µl)	Phenol reagt (ml)	Conc. sulphuric acid (ml)	O.D (λ490 nm)
1	0	1	1	1	5	0 .000
2	0.1	0.9	1	1	5	0.118
3	0.2	0.8	1	1	5	0.174
4	0.3	0.7	1	1	5	0.298
5	0.4	0.6	1	1	5	0.394
6	0.5	0.5	1	1	5	0.459
7	0.6	0.4	1	1	5	0.592
8	0.7	0.3	1	1	5	0.623
9	0.8	0.2	1	1	5	0.713
10	0.9	0.1	1	1	5	0.809
11	1	0	1	1	5	0.922

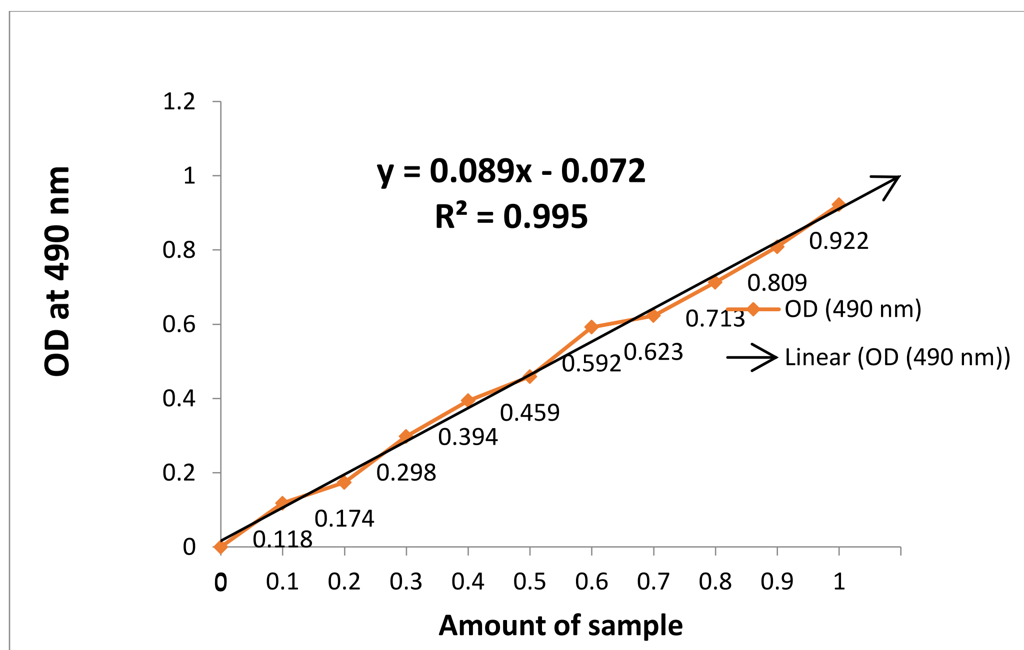

FIG. 8. **Standard curve showing the estimation of trehalose by phenol method.**

Results

i. The soluble protein present in the haemolymph of *Antheraea mylitta* has been depicted in table 2.0. In the present study total soluble protein from the haemolymph of *A. mylitta* was investigated using Lowry method (1951). The result showed the lower amount of protein content in the haemolymph. The amount of total soluble protein varied

from 37.514 μg to 38.366 μg indicating utilization of soluble proteins in the synthesis of silk filament for the formation of cocoon around itself.

ii. After comparing with the standard prepared from marker proteins the molecular weight of fibroin was calculated as approx 195 kDa to 198 kDa and molecular weight of Sericin was calculated as approx 65 kDa to 70 Kda.

iii. The trehalose concentration in the haemolymph of *Antheraea mylitta* is shown in table 4.0. The value of total sugar varied from 0.4523 μg to 0.4477 μg.

iv. Trehalose estimation was done by subtracting the mean OD of the untreated sample from the mean OD of treated sample (sample digested with trehalase enzyme).

Mean OD of digested, Mean OD of undigested difference in OD.

Sugar sample: 0.457, 0.412, 0.045.

In this study estimation of total sugar has been made using spectrophotometric Anthrone method. By adopting Anthrone method total sugar varied from 0.4523 μg-0.4477 μg.

Discussion

Tasar silk worm is a wild variety cultivated in natural conditions of the forests. The life cycle of this silkworm is controlled by the climatic conditions. Depending upon prevailing climatic conditions there are 44 eco-races found in India. Kumari and Roy (2011a) have studied the phenotypic and behavioural differences of eco-races of Tasar silkworm *Antheraea mylitta* Drury in relation to the environmental factors. Attempt has been made to study on some aspects of the identification of nutritionally efficient silkworm their metabolic rate and their sustainable development for the energy resources. They concluded that tropical Tasar silkworm may be a suitable and model species needed for sustainable development for the energy resources of the rural areas. Roy studied sustainable development a case study of Santhal Parganas (Jharkhand) by using wild variety of Tasar silkworm *Antheraea mylitta* and concluded that by cultivating this species both long and short term environmental management and conservation of the local ecosystem will be done. Taking such objectives, the present study of the "Biochemical analysis of haemolymph of *Antheraea mylitta*" has been undertaken. In the present project work an attempt has been made to investigate the total soluble protein (μg), SDS-PAGE Analysis for molecular weight determination of sericin and fibroin of silk filament and trehalose estimation using Spectrophotometric Anthrone and Phenol method has been performed on the pupal stage of tropical wild variety of the eco-race of *Antheraea mylitta* D. It is interesting to note that a close antagonistic relationship between total soluble protein and trehalose content has been observed. The silk fibre is a protein synthesized by a silk gland cells and stored in the lumen of the silk gland subsequently it is converted into the silk fibres. Therefore, silk gland is a bio-factory and silk is a biomaterial having enumerable applications into multifaceted fields. When the silkworm secretes the liquid silk during the spinning, it passes through anterior silk gland to be expelled out through the spinneret, opening through which the silk is passed out. The quantity and the nature of sericin are of fundamental characteristics in conferring distinct traits to the cocoon. Sericin protein is useful because of its special property such as resist oxidation, antibacterial, UV résistance, absorb and release moisture easily inhibits activity of tyrosine and kinase. Sericin is chemically a non-filamentous protein and contains also other natural impurities like fats and waxes in organic salts and colouring materials. Sericin is a hydrophilic. It is removed from fibroin during the silk manufacturing process for making lustrous and to remove the sericin, as waste product. Recently, these seri-waste products and seri-by product are used as a value-added product. After degumming the left over is fibroin made up of 2-chains. Fibroin is well known for water absorbency, dyeing affinity, thermo-tolerance and prevention of UV radiation. The differences between sericin and fibroin

indicate that in sericin more amino acids with non-polar side chain while in fibroin more amino acid with acid side chains are present. Another difference between wild and domestic silkworm sericin is that in former amino acid with non-polar side chain and in latter amino acid with polar side chain in sericin are found. Fibroin has higher amount of alanine, glycine and serine. A small amount of cysteine residue gives us very small amount of sulfur in the fibre and these have acid side chains. Fibroin is the principal water insoluble protein (i.e. 78% the weight of a raw silk). In the present study, the total soluble protein was estimated of the haemolymph of *Antheraea mylitta* and the value ranged from 37.514 µg to 38.336 µg. The SDS-PAGE analyses have clearly showed the molecular weight of sericin and fibroin present in the Tasar silk filament. There is striking differences between the band of the sericin and fibroin. In sericin, the molecular weight varied between 65 kDa-70 kDa while in fibroin the molecular weight varied from 195 kDa-198 kDa. The total haemolymph soluble protein content of *Antheraea mylitta* progressively decreased as the development proceeded and this might be due to utilization of protein during the silk synthesis in the late 5 thinstars. In this study estimation of trehalose has been made using spectrophotometric Phenol methods. By spectrophotometric Phenol method the total sugar varied from 0.4523 µg to 0.4477 µg. Interestingly in the present study a close relationship was observed in soluble protein content of haemolymph and trehalose concentration. Total soluble protein content and trehalose during earlier instar increased simultaneously but after reaching 4th instar the protein content decreased. However, trehalose concentration was decreasing from 1st-5th instar and in pupal stage the value is also at minimal level. The present work embodied some explanation to find out the relationship between total soluble protein trehalose and molecular weight of sericin and fibroin. It is well established that haemolymph of these insects is a store house of various kind of proteins and enzyme. By using electrophoretic method, a number of proteins have been identified in the haemolymph. One of the criterions to know the index of suitability of silk filament is the determination of the molecular weight of the silk fiber which is made from the sericin and fibroin. SDS-PAGE along with molecular weight marker were subjected to electrophoresis. In the present usually SDS protein was revealed. Mobility of marker proteins band was plotted against log molecular weight and on that basis straight line was drawn and from that it came out that the molecular weight of the protein bands of sericin was 65 kDa to 70 kDa while for the fibroin 195 kDa to 197 kDa. The result indicated that the protein content in silk fibre was quite high. Thus, the SDS-PAGE along with molecular weight determination using electrophoresis acts as a biomarker for the evaluation for the quality of silk fibre. Carbohydrates are a major fuel of Tasar silkworm for the growth and development and they derived mostly from the diet. The silkworm *Antheraea mylitta*, conserve sufficient quantity of energy during larval development for utilization during pupal and adult stages. Trehalose is the major and metabolically active non-reducing disaccharides present haemolymph which is synthesized in fat body. It is the reservoir for most of the biomolecules that is required for the nearly every physiological activity of the insect. Thus, the change in the composition of haemolymph reflects the morphogenic and biochemical changes taking place in insect tissue. The insect haemolymph perform several functions such as immunity, transport, storage etc. In the present study, an attempt has been made to investigate the standing state of the haemolymph of *Antheraea mylitta*. Trehalose is major disaccharides in the haemolymph. Its active biosynthesis occurs in the fat body and rapid utilization during silk synthesis in the last larval instar of the silkworm. The level of trehalose in the haemolymph reflects the level of carbohydrate in the body as well as physiological status of the insects thus, the level of trehalose act as an index of the physiological status of the insects. The level of trehalose is found to be the lowest in the pupal stage of the *Antheraea mylitta*; this may be due to the fact that the pupal stage is an inactive, no feeding, diapauses state which is surrounded by cocoon. The lowest level of trehalose in pupa is due to the consumption of carbohydrate. The increase in the trehalose level is associated with the feeding of leaves of the host plants. The higher level of carbohydrate is associated with the higher rate of food consumption of larval stage resulting in the

increased absorption of glucose through the digestive tract to cope up with the increased physiological activity. The lower level of trehalose in the pupal stage indicating its greater utilization to furnish fuel for active synthesis of silk and other physiological process like spinning Further, it may also be associated with the decreased food intake during the last part of the 5th instar larvae. Hirano and Yamashita have shown a correlation between the utilization rate and turnover rate of haemolymph trehalose during metamorphosis in *Bombyx mori*. Thus, present results are in accordance with the earlier findings of Saito, 1963 suggesting the existence of a possible homeostatic mechanism in the silkworm to regulate the trehalose level in the body. In Tasar silkworm trehalose unlike soluble proteins acts as indirect factor in the evaluation of the spinning and silk synthesis. In the present study, a very close relationship between trehalose level and total soluble protein was observed. In pupal stage both biochemical profiles were observed at their closely minimal level. Tasar silkworm is the wild variety and only propagated in open under natural conditions. Recently some attempts have been made to semi-domesticate this species under controlled conditions to avoid vagaries which interferes with the activities as well as the silk traits of the Tasar silk. The present study relating to total soluble proteins, SDS-PAGE for molecular weight determination of sericin and fibroin and trehalose level estimation will add some knowledge on amelioration of quality of Tasar silk as well as for giving clue for making it fully domesticated. Such traits will be achieved after manipulation of biochemical, architecture of the silkworms.

Conclusion

While searching the literature on the "Biochemical analysis of haemolymph of *Antherea mylitta*" information on the total soluble proteins, quantitative and qualitative analysis of amino acids, electrophoretic studies, SDS-PAGE etc are scanty. All these biochemical parameters are pertinent in amelioration of silk production and needed in the improvement of the quality and quantity of the Tasar silk. Considering all these facts in mind, the present study on the "Biochemical analysis of haemolymph of *Antheraea mylitta*" has been undertaken. The reduction of rural poverty continues to be a paramount goal of the developing countries like India as the majority of the poor population still resides in the country-side. The World Bank, for example, estimates that more than 70% of the world's poor live in rural areas. So far, various strategies have been pursued to address this concern and among the major ones is rural employment creation. The agriculture sector, however, has been contending with a number of factors that have limited its potential for generating new jobs in rural areas. Those factors may include the small land holding size, insufficient capital and investment incentives, the inadequate farm infrastructure, limited market and stagnant prices of agricultural products. It is, therefore necessary to focus on a broader spectrum of the rural economy. The establishment of rural based industries like sericulture, in particular, can be very effective in creating new job opportunities and providing supplemental income. Being a rural agro-based labour intensive industry this sector can also play vibrant role in checking migration from rural to urban areas. In this project work, the present status of the sericulture industry in India, its trends, position in global sericulture and science and technological achievements have been reviewed. Besides, some critical issues like potentiality of the sector in national economy, rural development, women empowerment and employment generation have been identified. An attempt has been made to draw a strategic model to strengthen and promote sericulture industry in India to enhance productivity and quality of silk etc. This project would be helpful in recognizing the potential, strength and challenges of the sericulture industry in India so as to formulate certain policies and measures for socio-economic development. Tasar silk worm is a wild variety cultivated in natural conditions of the forests. The life cycle of this silkworm is controlled by the climatic conditions. Depending upon prevailing climatic conditions there are 44 eco-races found in India. An attempt has been made to study the phenotypic and behavioural differences of eco-races of Tasar

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