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Protein profile of Native population of *Antheraea assamensis* Helfer (Muga Silkworm) of Assam, India

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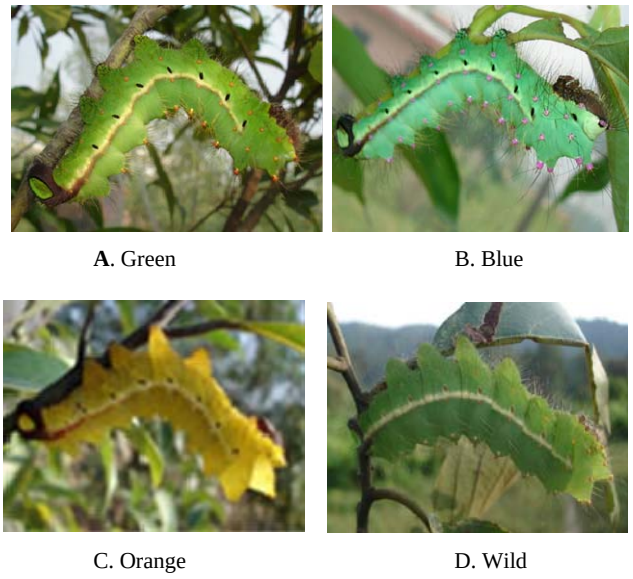
Abstract
The present study seeks to investigate the comparative estimation and electrophoretic profile of haemolymph proteins among the different semi domesticated morphs (green, blue, orange) and the wild (bivoltine) morph of *Antheraea assamensis* Helfer in order to investigate their differential gene expression. The proteomic approach was utilized to investigate the proteome of the haemolymph of early, mid and late stages of 5th instar (larval stage) and to improve the understanding of their important bioprocess and gene expression situation. The results suggest that the quantitative approach is useful to arrive at a conclusion of genetic variation among the morphs. SDS-PAGE electrophoresis of protein associated with haemolymph revealed separation of 16 distinct protein bands among which some of them represent the storage and immunity related proteins.

Keywords: Protein profile, semi-domesticated, silkworm, *Antheraea assamensis*.

1. Introduction
Proteins are among the most complex of all known chemical compounds and also the most characteristic of living organism (Chen, 1985) [1]. The protein component of insect haemolymph comprises functionally and structurally heterogeneous arrays of macromolecules (Wyatt and Pan, 1978) [13]. The insect haemolymph protein are also considered as storage protein and in a number of insects species reaches a maximum during the final larval instar (Chipandale and Kilby, 1969) [2].
Allopatric populations occur in *Antheraea assamensis* with distinct coloration viz. green, blue, orange and yellow in their larval stages (Thangavelu *et al.*, 1988) [12]. These varieties are multivoltine, produces 5-6 generation in a year. Evidence shows that a wild stock with diapausing characters is available in dense forest which often comes down to the vicinity of human habitation by the attraction of female moth (Chowdhury, 1981) [3]. Due to its transitional nature between domestic and the wild, it shows a wide range of morphometric variation. The colour morphs and the wild counterpart vary in their phenotypic characters depending upon the alternation of generation, host plant selection or seasonal changes. Moreover, according to habitat status the animal may change their character (Singh & Sen, 2001) [11]. Estimation on the genetic diversity and relationship between the germplasm collections has been proved to be useful for facilitating efficient germplasm collection and management with the help of various molecular markers.
The haemolymph protein of the silkworm undergoes a complex sequence of changes which are synchronized to the morphological alterations which takes place during metamorphosis. Heritable or genetic changes in the non-enzymatic protein have also been reported. Some proteins namely albumin, storage protein, lipoprotein in insects are reported to the species specific (Wang and Haunuland, 1992) [14]. The present investigation in an attempt to investigate the proteome of the haemolymph of four morphotypes of *Antheraea assamensis* for understanding their difference if any.

2. Materials and methods
2.1. Experimental animal
The experiments were conducted on the larvae of four morphotypes (morphs) [Plate-1] of *Antheraea assamensis* during the period from 2012-2014. The seed cocoons of green and blue morph were collected from various parts of muga growing areas of Assam and Meghalaya. The orange morphs were collected from the Barpeta district (Howly) of Assam, after the

reporting in 1972 at Goalpara district, Assam, India (Chowdhury, 1981) [3]. The wild morphs were collected from the forest in the diapausing cocoon and larval stage from different border area of Assam, Arunachal Pradesh and Meghalaya. To obtain the larva, morphs were reared separately at the IASST (Institute of Advanced Study in Science and Technology, Guwahati, Assam) garden for their multiplication. Som (*Machilus bombycina* King) plants were used for feeding the worms throughout the experiment.



A. Green B. Blue

C. Orange D. Wild

Plate 1: Four morphotypes of *Antheraea assamensis* Helfer (A. Green, B. Blue, C. Orange colormorph and D. Wild counterpart)

2.2. Collection of Haemolymph

Haemolymph was collected from early (E), mid (M) and late (L) part of feeding stages of 5th instar larvae by giving a slit under the ventral region of foreleg in the autoclaved prechilled eppendorf tube and rinsed with 3% PTU (phenyl thiourea) under laminar flow. The collected haemolymph were centrifuged immediately to get rid of haemocytes and stored at –20 ^{0}C for further experiments.

2.3. Biochemical analysis

Estimation of haemolymph protein was done by Folin–Ciocalteau's method described by Lowry *et al.* (1951) [4]. The statistical calculations were performed by using SPSS software for determination of significant differences (at 0.05% ANOVA) among the morphs.

2.4. SDS-Electrophoresis

Molecular weight of haemolymph protein of the silkworm was ascertained by adopting 10% SDS-PAGE using the method of Laemmli (1970) [5]. Haemolymph samples were diluted with sample buffer containing 5% mercaptoethanol and heated at 100 ^{0}C for 3 min before application to the gel 20µl of each was used for electrophoresis. The electrophoresis was performed at 20mA for 6 – 7 hrs. Marker proteins (molecular weight 97 to 14.2 kda) were also loaded in appropriate concentration.

2.5. Gel Visualization & Characterization

After a brief wash in distilled water, the gel was emerged in fixing solution for 2 hrs and agitated steadily on a shaker. The fixing solution was poured out and the gel covered with Coomasie blue solution (R-250) for 6 to 8 hrs. After pouring off the staining solution, the gel was destained for 2 hrs with destaining solution. The proteins fractioned into band are seen colored blue. The gel was then placed under VDS-image master for scanning and molecular weight calibration.

3. Results

3.1. Haemolymph Protein concentration

The result of the total protein concentration has been shown in Table-1. The estimation of the haemolymph protein content showed the significant variation among the morphs in E, M and L stages of the 5th instar larvae. The present study revealed that the protein content increased gradually from early stage and attained highest at late stage of each morph. Among the morphs, the highest protein content was observed in the wild morph while the lowest content observed in the blue morh in all the stages.

Table 1: Showing the protein content of Haemolymph in different stages of 5th instar larva. The results are mean ±SD of 10 replicas Values having different subscripts differ significantly ($p<0.05$).

	Total soluble protein(mg/ml)		
	Early SD	Mid SD	Late SD
Green	10.04a±2.08	40.53ab±4.79	70.20ab±4.77
Blue	9.60a±2.01	35.24a±7.49	65.67a±7.55
Orange	9.81a±0.00	39.14ab±0.00	68.32a±0.00
Wild	14.05b±0.43	45.15b±0.85	77.92b±1.41

3.2. SDS-Electrophoresis/Protein Image Acquisition and Analysis

A total number of 22-26 bands of haemolymph proteins were identified during this study as shown in fig.1. As the molecular markers used were from 97 to 14kda, we could determine the weight of proteins occurring in this range.

The expression was highest in wild morph with 26 protein bands and lowest in the orange morphs. The band analysis revealed almost similar in green and blue morphs.

It was observed that the concentration of storage protein was much more in wild (molecular weight 89.571 and 81.429) in compare to other morphs.

We were able to detect 16 different protein bands. The expression is highest in blue mature with 15 protein bands and lowest in the orange. The band analysis and pixel intensity revealed that 60-70 kDa protein is absent in the wild variety as well as blue mature and the early stages of the green. The low molecular weight 11-12 kDa protein is also absent in the wild variety. The present study revealed that the orange variety is the intermediate between green and blue and more closer evolutionary relation to the green. The wild variety has close relationship with the green and more closer to the blue morph.

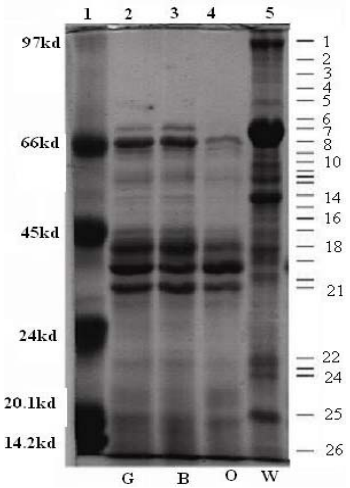


Fig 1: SDS-PAGE of different morphs at Matured stage (5th instar).Lane 1-Standard, 2-Green, 3-Blue, 4-Orange, 5-Wild.

4. Discussion

The concentration of protein at different phase in E, M and L of 5th instar larvae increased gradually which is in conformity with the report Pant and Morris (1972) [10]. As the food consumption at this stage is highest in silkworm, the accumulation of protein also increased accordingly. The protein content may be correlated to the body weight as has been noticed in this present observation. Highest protein content of wild reveals that phenomenon.

Key elements of classical proteomics are the separation of proteins in a sample using SDS-PAGE. The cell associated protein showed a complex pattern of around 26 staining band ranging from 97-14 kda. Although there were minor quantitative differences were observed in the protein in some bands among the morphs, the intensity of some of the bands were differ significantly. These differences in their quantitative and qualitative nature of protein attributes that they ultimately control the silk yield (Garno, 1993) [6]; Hirobe, 1968) [7]. The presence of bands specific to each morphs and to particular genotypes indicate their potential use for marker-assisted breeding and varietal identification.

Proteomics is a large-scale study of the gene expression at the protein level, which ultimately provides direct measurement of protein expression levels and insight into the activity state of all relevant proteins. Key elements of classical proteomics are the separation of proteins in a sample using SDS-PAGE. In order to conclude the study, it felt necessary to make clear whether similar patterns are evident in other more closely related groups.

In silkworm larvae, the protein content increases up to the development into 5th instar larval development (Pant and Morris, 1972) [10]. Haemolymph of insects contains many proteins; but along with normal haemolymph proteins some immunity related proteins viz. apolipoprotein of 18kda are also present in them as well as some induced proteins are also formed.

The quantitative and qualitative analysis of protein among the four morphotypes reveals a relationship as green and orange morph is very closer, blue is distant group and the wild is far distant from the others which confirms the result of wing characters of morphotypes of *Antheraea assamensis* (Nath and Devi, 2009) [9].

5. Conclusion

Proteomic approach was utilized in this study to investigate the proteome of the haemolymph during growth and development, and to improve the understanding of important bioprocess and gene expression situation (Nabby-Hansen *et al*., 2001) [8]. There is a relation in between the protein content and electrophoretic pattern of haemolymph of *Antheraea assamensis* morphs. The biochemical analysis by electrophoresis has shown the difference among the morphs. The electrophoresis result will not only provide a method for direct measurement of protein expression levels and insight into the activity state of all relevant proteins, but also the elucidation of gene function and regulation. The proteins, especially the enzymes, are differentially expressed during the growth and development of silkworm. This has implications for the physiological and biochemical proceedings and the gene regulation in the silkworm.

6. Acknowledgement

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