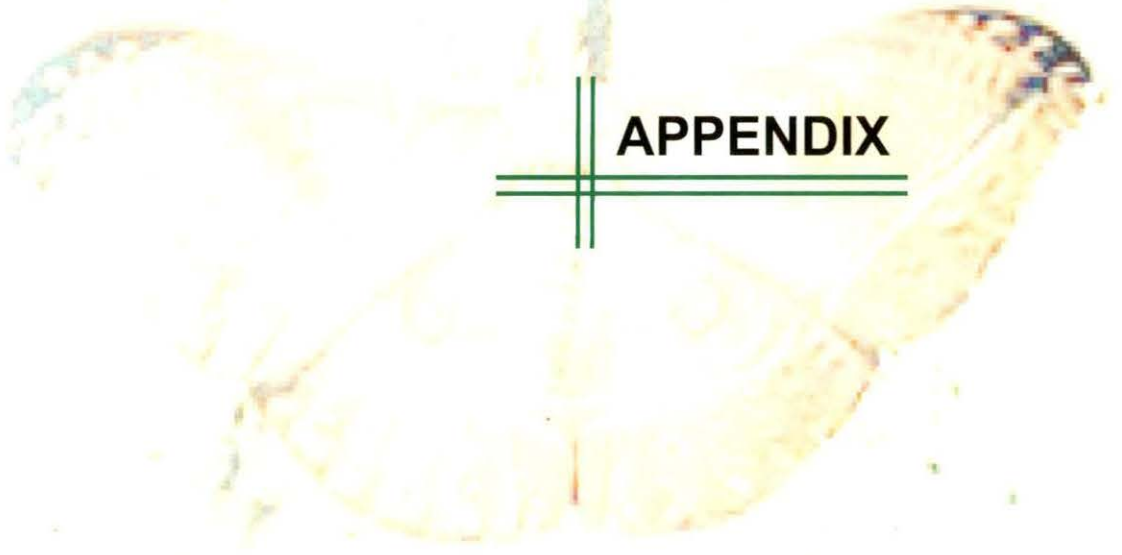
APPENDIX



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Biochemical profiles of haemolymph of the muga silkworm, *Antheraea assama* Ww (Lepidoptera : Saturniidae) reared on two major host plants and their impact on cocoon characters

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ABSTRACT : A comparative study was undertaken with respect to protein, lipid and carbohydrate contents of haemolymph and body water content of the late-aged third, fourth and fifth instar larvae of the muga silkworm *(Antheraea assama* Ww) after rearing on two major host plants, Som *(Persea bombycina* Kost) and Soalu *(Litsaea polyantha* Juss). The study was undertaken in Coochbehar district of West Bengal, which is a recently introduced non-traditional area for muga silkworm culture. The protein and carbohydrate contents were always higher in the larvae reared on soalu plant. A reverse result was obtained in case of lipid. Larval body water content had very little relation to the diet. Cocoon weight and shell weight did not differ significantly when reared on the two host plants, but silk ratio was significantly higher in the case of som plant.

Keywords : Muga, *Antheraea assama,* haemolymph protein, lipid, carbohydrate.

Introduction

Antheraea assama Westwood (Lepidoptera : Saturniidae), the muga silkworm, is a multivoltine sericigenous insect reared on natural host plants Som *(Persea bombycina* Kost) and Soalu *(Litsaea polyantha* Juss). Quality of leaf directly influences the quality of silk and fecundity of the silkworm (Sarkar *et al.,* 1992; Sinha *et al.,* 1993, Chaluvachari and Bongale, 1996). Proteins are considered important biochemical parameter because of their crucial role in the development, morphogenesis and in many of the intermediary metabolic pathway of the insect (Kar *et al.,* 1994). Several information are available on the haemolymph proteins of *Bombyx mori* (Lauffer, 1943; Wyatt and Pan, 1978; Ogawa and Tajo, 1981; Ray, 2000). Preliminary information on protein contents in larval and pupal haemolymph of the tropical tasar silkmoth, *Antheraea mylitta* (Sinha *et al.,* 1985) and of the muga silkmoth *A. assama* (Sharma *et al.,* 1995) is also available. Goel *et al.* (1988) reported the changes in lipid contents during embryonic development of *A. mylitta.* Changes in lipid content during larval period may be due to differential metabolic activity of the larval instars (Sinha *et al.,* 1992). Preliminary information of the lipid contents in *A. assama* is available from the works of Bordoloi and Hazarika (1992, 1998) and Hazarika *et al.,* 1995). The haemolymph carbohydrate content was worked out in this species by Dutta *et al.* (1997), who emphasized on the influence of carbohydrate content on high metabolic activity of the larvae. However, no information is available on the profiles of protein, lipid and carbohydrate contents of *A. assama* reared on two major host plants and their impact on the cocoon characters in any non-traditional area.

An attempt was therefore, made to investigate the protein, carbohydrate and lipid contents of haemolymph and water content of larval body of the late-age third, fourth and fifth larval instars of *A. assama* reared on the two major host plants which have been introduced in non-traditional region in the district of Coochbehar, West Bengal.

Materials and Methods

Disease free eggs of *A. assama* were collected from the Muga Basic Seed Farm (Directorate of Sericulture, W.B.), Khagrabari, Coochbehar. After

hatching, the larvae were reared on som and soalu host plants in two prime seasons (Shankar *et al.*, 2002), April-May and September-October, 2003. The haemolymph was collected from the third, fourth and fifth instar larvae immediately after cessation of feeding following the method of Chen (1961). A pinch of phenylthiourea and EDTA was added to avoid melanisation and clotting. Samples after centrifugation were subjected to Lowry's method (Lowry, 1951) for protein estimation, to Anthrone method (Plummer, 1979) for carbohydrate estimation, and to colorimetric method (Bragdon, 1951) for lipid estimation. Moisture content of larval body was determined following Paul *et al.* (1992). The data of two prime seasons were pooled and analyzed statistically.

Results

Protein content : Irrespective of the host plant the total protein contents of the three larval instars differed significantly, the content of the fifth stage larvae was more than 5 times and 4 times that of the third and fourth stage larvae respectively. On the whole, the protein content was significantly higher in all the instars when reared on soalu plant than on the som plant (Table 1).

Lipid content : Lipid content also increased with the advancement of larval instars, the differences between two successive instars were nearly 3 times. But the lipid contents were significantly higher in case of som as the host plant except in the third instar where there was very little differences with respect of the two host plants (Table 1).

Carbohydrate content : The carbohydrate content was also significantly higher in the three instars when reared on soalu plant. Among the instars the trend of differences was like that of the protein contents (Table 1).

Table 1. Means (±SE) haemolymph protein, lipid and carbohydrate contents (mg/ml) and larval body water content (%) of *Antheraea assama* reared on som and soalu plants.

Larval Instar (Sample No.)	Total Protein		Total Lipid		Total Carbohydrate		Water Content	
	Som	Soalu	Som	Salu	Som	Soalu	Som	Soalu
Third (30)	7.03±0.34	9.40±0.37	2.89+0.16	2.19±0.09	3.34±0.25	5.01±0.18	90.52±0.28	88.56±0.36
Fourth (30)	9.51±0.25	10.60±0.18	7.36±0.40	5.88±0.38	5.23±0.16	6.06±0.14	91.38±0.29	91.04±0.27
Fifth (3)	40.56±2.00	49.29±1.58	26.71±1.13	20.48±0.60	15.09±0.23	20.05±0.46	86.62±0.47	87.92±0.46

CD at 5%	Leaf	0.48	0.64	0.33	0.49
	Instar	0.37	0.50	0.26	0.40
	Leaf X Instar	0.73	1.00	0.52	0.77

Table 2. Cocoon characters of *A. assama* reared on som and solau plants.

Host Plant (Sample No.	Weight in gram (±SE)			SR%
	Single 5th instar Larval weight	Single Cocoon Weight	Single Shell Weight	
Som (30)	7.99±0.65	5.22±0.35	0.42±0.02	8.09±0.30
Soalu (30)	9.26±0.95	5.97±0.28	0.39±0.06	6.57±0.46
CD at 5%	0.32	0.56	0.05	0.38

Larval body water content : Larval body water content has no relation to the host plant. But there were significant differences among the instars. The lowest water content was recorded in the fifth instar and highest in the fourth instar (Table 1).

Cocoon characters : Larval weight was significantly higher in larvae reared on soalu (9.26 g) than in larvae on som (7.99 g) plant. Cocoon weight was significantly higher in case of larvae reared on soalu plant (5.97 g). Shell weight though having non-significant differences, was a little higher (0.42 g) in case of som as the host plant (compare to 0.39 in soalu). Shell ratio percentage (it is the percentage of the ratio of shell weight to cocoon weight) was higher in the cocoons obtained from som leaves as diet (Table 2).

Discussion

Increased biochemical contents in fifth instar larva, more than three times than those of the fourth instar, were due to higher accumulation of resources from much higher quantity of food consumption (Annual Report : CSB, 1987-88) during late fifth instar stage which is the crucial period regarding energy storage for metamorphosis from larva to pupa and for the formation of cocoon as well as for gametogenesis especially in female (Mullins, 1985). Protein and lipid contents have direct relevance to the accelerated metabolism of silk gland, and to the reproductive allocation, respectively. The increased carbohydrate content is required as the energy source for increased metabolism during metamorphosis as well as for gametogenesis. These results have clear conformity to those reported by Sinha *et al.* (1992). Unni *et al.* (1997) and Bashamohideen and Ameen (1998).

It was found that the protein and carbohydrate contents of haemolymph of larva fed with soalu leaves were higher though its reflection on shell weight and shell ratio was not correspondingly higher. Conversely, in case of larvae fed with som leaves though contains lower protein and carbohydrate contents in haemolymph, the cocoon traits are better.

Acknowledgements

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STUDIES ON BACTERIOSIS OF MUGA SILKWORM *Antheraea assama*Ww (Lepidoptera: Saturniidae) IN THE COOCHBEHAR DISTRICT OF WEST BENGAL

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ABSTRACT

The silkworm, *Antheraea assama* Ww. (Lepidoptera: Saturniidae) is a wild bioresource that produces precious golden muga silk. It is endemic to northeast Indian places of which Bramhaputra valley of Assam and Meghalaya are specially worth mentioning. An attempt has been made to rear this insect semi domestically and to extend its commercial rearing in non-traditional region, the district of Coochbehar, West Bengal. The agro-ecological contiguity of Coochbehar to northeastern states of India makes the district promising for rearing. A study undertaken during 2001-'03 on the prevalence of bacterial diseases of muga silkworm in this non-traditional region has revealed that the incidence of bacteriosis (bacterial flacherie) is very high in this region. Several gram positive and gram-negative bacteria recovered from worms suffering from bacteriosis, belonged to cocci and bacilli. Of the six pathogenic genera identified so far, four belonged to Enterobacteriaceae. The identified genera are *Klebsiella, Pseudomonas, Proteus, Bacillus, Citrobactor* and *Providentia.* A few cocci recorded may be the secondary invaders.

Key words: *Antheraea assama,* muga silkworm, bacteriosis (bacterial flacherie).

INTRODUCTION

Antheraea assama Ww (Lepidoptera: Saturniidae) is a multivoltine wild silkmoth exclusive to India and endemic to the northern states particularly in the Bramhaputra valley of Assam, Meghalaya and adjoining hills. It has been introduced to the district of Coochbehar, West Bengal; the area has an agro climatic contiguity to the endemic region of Assam. Out of all the major causes, diseases of the worms affect cocoon yield. In *Bombyx mori* viral and bacterial flacherie causes about 70 per cent crop loss in Japan (Aruga and Tanada, 1971) and in India between 20 and 40 per cent (Chitra *et al.*, 1975). Bacterial flacherie or Bacteriosis alone is a serious concern in *B. mori* (Krishnaswami, 1978; Samson *et al.* 1990; Vanitha Rani *et al.*, 1994), causing a crop loss between 30 and 50 per cent (Chitra *et al.*, 1975; Vanitha Rani *et al.*, 1994; Dutta, 1995). The bacteria belonging to the genera *Bacillus, Streptococcus, Serratia, Staphylococcus* and *Proteus* cause bacteriosis in *B. mori* (Krishnaswami *et al.*, 1973).

In Assam *A. assama* also suffers frequently from bacteriosis (Senapati *et al.*, 2001 and Senapati *et al.*, 2002) but no bacterium has been identified for the disease. Choudhury *et al.* (2002) made preliminary report on the existence of the bacterial strain AC-3 associated with bacteriosis. In fact detail information on bacteriosis in *A. assama* is lacking. This communication is the result of a preliminary study of bacteriosis in *A. assama* in the non-traditional area, the Coochbehar district, West Bengal.

MATERIALS AND METHODS

Bacteriosis affected fourth and fifth instar larvae of *A. assama* were collected from five distantly inhabited farmers, one each from Coochbehar, Tufanganj and Mathabhanga and the remaining two from Dinhata Subdivision. Bacteriosis affected worms were diagnosed by clinical symptoms narrated by Thangavalu *et al.* (1988), Chishti *et al.* (1991) and Sanakal *et al.* (1996).

Body surface of the larvae was sterilized with 70 per cent ethanol (Vanitha Rani *et al.*, 1994), and then homogenized in insect ringer solution. The homogenate of each larval instar was incubated overnight at 37°C in four culture media such as nutrient broth, nutrient agar, MacConkey agar and blood agar. Bacteria from morphologically identical and uniform colonies were subjected to subculture. Bacteria from both original plates and from subcultures were stained with Gram's stain and spore stain. The method of culture, identification of bacteria and their biochemical characterization were carried out following Bergey's manual (Hardie, 1986) and Collins *et al.*

(1989). Healthy larvae were used as control for the culture by the same protocol.

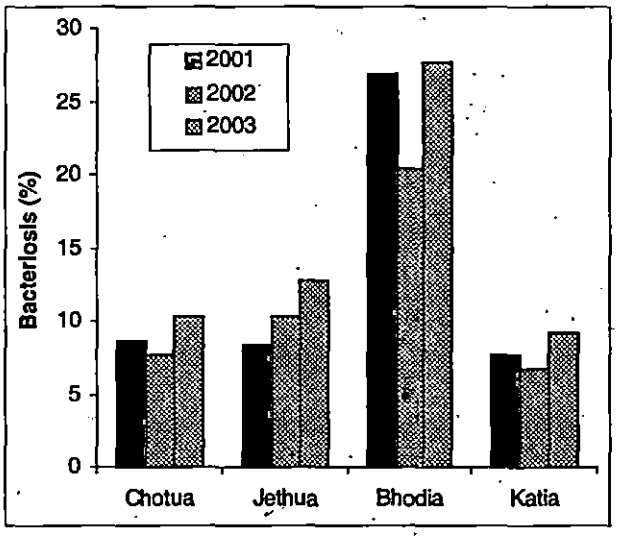

Fig.1. Incidence of bacteriosis in Coochbehar district of West Bengal

Table- 1: Incidence of bacteriosis (%) in Coochbehar district of West Bengal (mean±SD)

	Incidence of bacteriosis (%)		
Month (Crop)	**Year: 2001**	**2002**	**2003**
Mar-Apr (Chotua)	8.61 (±1.07)	7.69 (±1.23)	10.28 (±1.59)
May-Jun (Jethua)	8.38 (±1.58)	10.30 (±2.00)	12.72 (±1.71)
Aug-Spt (Bhodia)	26.86 (±5.24)	20.42 (±3.05)	27.64 (±1.70)
Oct-Nov (Katia)	7.70 (±1.69)	6.78 (±1.55)	9.11 (±1.15)
CD at 5%	Crop-0.587	Year-0.508	Crop X Year- 1.711

RESULT AND DISCUSSION

The diseased larvae were identified by the symptoms of lose of appetite, sluggishness, progressively becoming flaccid and dark brown (Plate Ii), rapid palpitating dorsal vessel, vomiting brown fluid, soft and sticky faeces

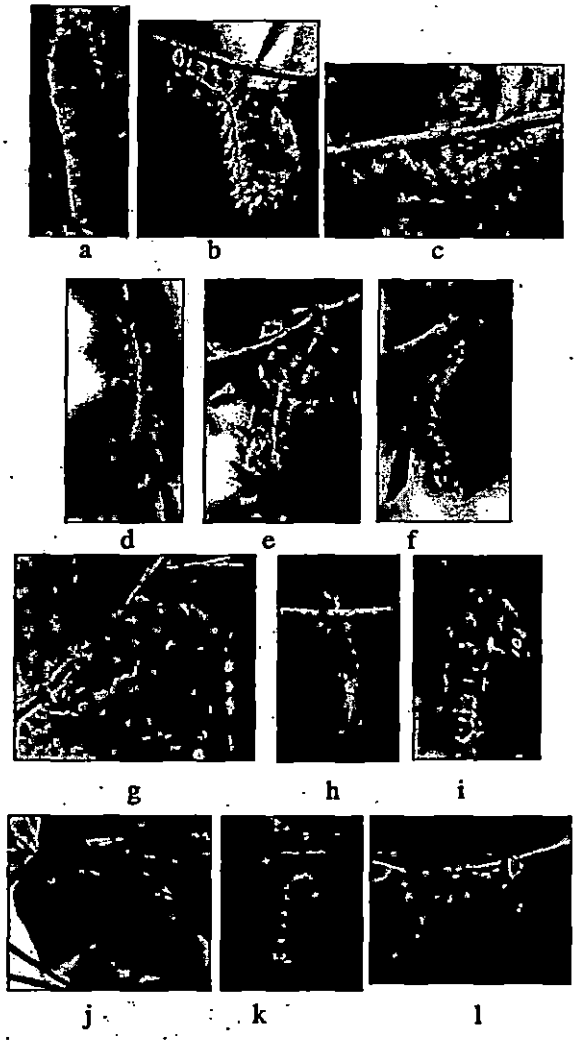

Plate I. Symptoms of worm suffering from bacteriosis compare to healthy.

a. Smooth skin of healthy worm. b. & c. Wrinkled skin of diseased worm.d. Exuvium shedding in healthy worm. e. & f. Exuvium shedding in worm suffering from bacteriosis. g. Clasping in healthy worm. h. & i. Clasping in diseased worm. j. Defecation and shape of faecal pellet of healthy worms. k. & l. Defecation and shape of faecal pellet of diseased worms.

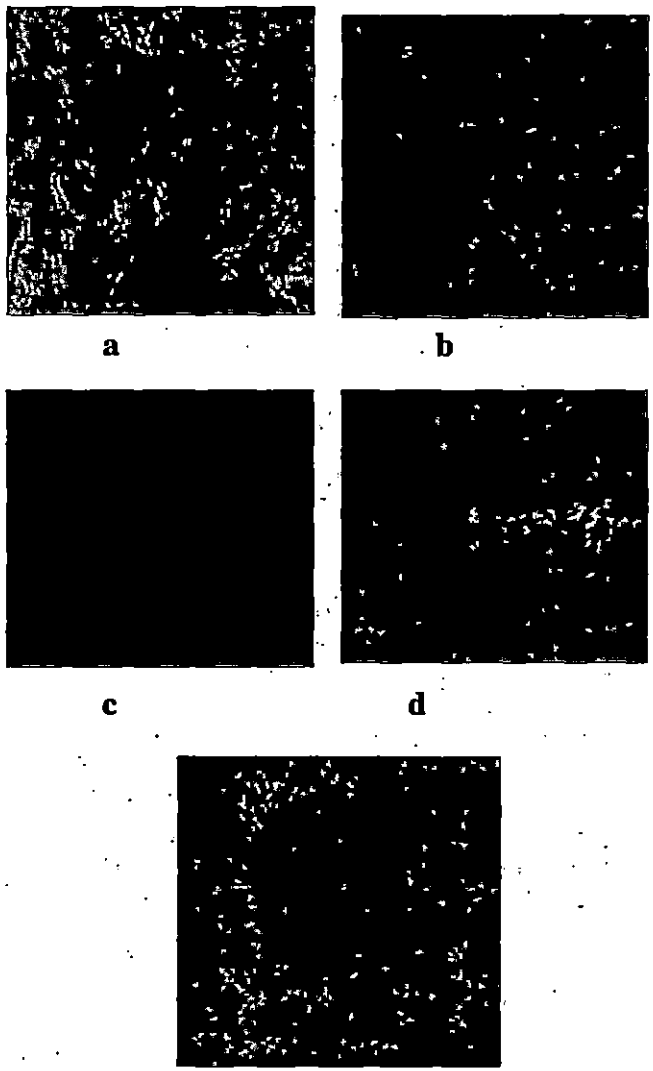

Plate II. Different types of bacterial pathogens.

a. Gram negative bacillus. b. Gram negative coccus.
c. Gram positive bacillus. d. Gram positive coccus.
e. Mixed population of gram negative and gram positive bacteria.

Table 2. Morphological and biochemical characteristics of the bacteria isolated from muga silkworms suffering from bacteriosis

Criteria	Group I	Group II	Group III	Group IV	Group V	Group VI
1. Morphology of the colony	Large flat haemolytic colonies in BA, yellow-green pigmented in NA	Mucoid colonies in BA & MA, stringy type growth	BA(+), MA(+), flat colonies with serrated edge	Swarming growth over the surface of BA, distinctive smell in MA	NA(+), Flat cofonies in BA with greyish hue	Grey- white irregular colonies with wavy edges in BA, haemolytic
2. Microscopic examination	Rod, bipolar staining	Capsulated, straight rod	Rod shaped	Pleomorphic rod	Rod	Capsulated rod,
3. Gram stain	-	-	-	-	-	+
4. Spore stain	-	-	-	-	-	+
5. Biochemical characteristics:						
a. Citrate utilization	+	+	+	+	+	-
b. Oxidase	+	-	-	-	-	-
c. Catalase	+	+	+	+	+	+
d. Gelatin test	+	-	-	+	-	-
e. Motility	+	-	+	+	+	+
f. Indole	-	-	-	+	+	-
g. Urease activity	-	+	+	+	+	-
h. H_2S production	-	-	+	+	-	-
i. Gas production	-	+	+	+	-	+
j. Methyl red	-	-	+	+	+	+
k. Voges proskauer	+	+	-	+	-	+
l. Triple sugar utilization						
i) Lactose	-	+	+	-	-	+
ii) Glucose	-	+	+	+	+	+
iii) Sucrose	-	+	-	+	+	+

Note: NA- Nutrient agar, MA- MacConkey agar, NB- Nutrient broth, BA- Blood agar, H_2S- Hydrogen sulphide.

(Plate I k & l), improper shading of exuvium (Plate I e & f), spasms and tremor of body, paralysis, sudden collapse and death.

On an average 11-15 per cent mortality/year due to bacteriosis was recorded in the field (Table-I). The incidence was the highest in 'Bhodia' (Aug.– Sept.) crop. But the 'Katia' crop (Oct. – Nov.) was affected least.

The bacteria isolated belonged to cocci and bacilli (Plate-II). After biochemical characterization six groups (I – VI) of bacilli could be identified. The details about the groups in respect of their colonial, morphological and biochemical characters are furnished in Table-2. The groups I-V appeared blue and turbid in Koser citrate medium indicating alkaline reaction and utilisation of citrate as their source of carbon. After employing indole test of all the subcultures only the group V showed indole positive. In case of H_2S production test Group III and IV blackened showing positivity when cultured in Kligler iron agar (KIA) medium. Group I bacteria acquired deep purple blue colour within 10 second when placed in a filter paper socked with oxidase reagent. The groups I and IV released bubble of oxygen after placing them in 3 per cent H_2O_2 with a glass rod indicating the presence of catalase enzyme.

Result of morphological, staining and biochemical characterization indicate the existence of the genera *Pseudomonas, Klebsiella, Citrobactor, Proteus, Providentia* and *Bacillus* in the bacteriosis affected muga silkworm in Coochbehar district of West Bengal. In the bacteriosis affected mulberry silkworm, *Bombyx mori*, Chitra *et al.* (1975) obtained three species of *Proteus*, three species of *Pseudomonas* and two species of *Bacillus*. Enomoto *et al.* (1987) reported the existence of genera *Pseudomonas, Proteus, Bacillus* in septicaemia affected larvae of *B. mori*. Furthermore, both gram positive and gram negative cocci were found in the muga silkworms suffering from bacteriosis. Further studies were required to understand the pathogenesity of the identified bacteria in the different seasons in this non-traditional area.

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Comparative Study of Free Amino Acid Contents in the Haemolymph of Healthy and Diseased *Antheraea assama*Ww (Lepidoptera:Saturniidae) Larvae

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ABSTRACT

Comparative changes in the concentration of haemolymph free amino acids were estimated in healthy and diseased (bacteriosis) fourth and fifth instar muga silkworms, *Antheraea assama*Ww (Lepidoptera: Saturniidae) reared on two major host plants Som (*Persea bombycina* Kost) and Soalu (*Litsaea polyantha* Juss). The study was undertaken in a recently introduced non-traditional area. Seventeen free amino acids were recorded in the haemolymph of which nine were essential. The total free amino acid content was the highest in the fifth instar larvae reared on soalu plant. Irrespective of the host plants nonessential amino acids were quantitatively higher than the essential amino acids. There was a depletion of free amino acid content in the haemolymph of both the instars of diseased (bacteriosis) worms reared on som plant. An attempt was also made to correlate various biochemical significance of the amino acid profiles to the disease.

Keywords: Antheraea assama, haemolymph amino acids, bacteriosis.

INTRODUCTION

Antheraea assama Ww (Lepidoptera: Saturniidae) is an endemic wild silk moth of India, commercially reared semidomestically in northeastern states of India particularly in the Brahmaputra valley of Assam, Meghalaya and adjoining hills. In the Sub Himalayan terai region and adjoining plains of West Bengal, such as in the district of Coochbehar it grows quite well because of agro-ecological contiguity of Coochbehar to the northeastern states of India (Ray *et al.*, 2005). The muga silkworm is a multivoltine species reared on two major natural host plants, Som (*Persea bombycina* Kost) and Soalu (*Litsaea polyantha* Juss.). Quality of leaves directly influences the quality of silk and egg production in silkworms. The late larval stages of muga silkworm encounter outdoor environmental fluctuations, which may affect biochemical profiles of the haemolymph. The haemolymph is the medium of mobilization of the metabolites in insects. The changes of biochemical profiles may have a relation to disease susceptibility, which impairs silk yield. Therefore, it is necessary to explore the causal relation of diseases to the impact of major host plants and consequent haemolymph biochemical profiles of muga silkworm in this non-traditional region.

In *Bombyx mori* the loss in silk production due to bacterial diseases is 30-50% in a year (Chitra *et al.*, 1975; Vanitha Rani *et al.*, 1994; Dutta, 1995). Among the various diseases, the bacterial

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flacherie is of paramount importance (Krishnaswami, 1978; Samson *et al.*, 1990; Vanitha Rani *et al.*, 1994; Unni *et al.*, 2006). The higher level of free amino acid content in the haemolymph is associated with the healthy growth of larvae (Ito and Arai, 1967; Lazar and Mohamed, 1988). A series of information is available on the haemolymph free amino acid of *B. mori* larvae (Chen, 1962; Alieva and Filippovich, 1968; Aruttyunyan *et al.*, 1972; Inokuchi, 1972; Bose *et al.*, 1989 etc.). Sinha *et al.* (1988a) reported the profiles of free amino acids in haemolymph of the larvae of the tasar silkworm, *Antheraea mylitta*. Sharma *et al.* (1995) reported the seasonal variation of haemolymph free amino acid of *A. assama* reared on two host plants. Sinha *et al.* (1988b) showed the changes of free amino acid of pebrine infected *A. mylitta*. So far there is no report on the free amino acid profiles of the larvae of *A. assama* and any relation of the amino acids to the susceptibility to diseases. An attempt was therefore made to investigate the haemolymph free amino acid profiles of the fourth and fifth instar healthy and bacteriosis-diseased larvae of *A. assama* reared on the two major host plants, in recently introduced non-traditional region in the district of Coochbehar, West Bengal, India. Furthermore, the amino acid contents were assayed in only those diseased worms reared on som plant, as it is mostly used during commercial rearing.

MATERIALS AND METHODS

Source of larva: Matured healthy fourth and fifth instar larvae of *A. assama* immediately after gut purging were collected from the Muga Basic Seed Farm, Khagrabari, Directorate of Sericulture, Govt. of West Bengal. Diseased worms were collected from the fields of registered farmers of different subdivisions of Coochbehar district. Worms suffering from bacteriosis were identified by their clinical symptoms as described by Chishti *et al.* (1991), Sanakal *et al.* (1996), Thangavalu *et al.* (1988) and Govindan and Devaiah (1995). The worms were always collected at dawn.

Collection of haemolymph: The abdominal legs of healthy and diseased larvae were pricked with glass capillary tube and haemolymph was collected in ice-cold Eppendorf tubes. A pinch of phenylthiourea and EDTA was added to the sample to avoid melanisation (Cheung *et al.*, 1978) and clotting (Wheeler, 1963).

Preparation of haemolymph samples: In order to remove the haemocytes and tissue debris, all the haemolymph samples were centrifuged at 3000g for 2 minutes (Kar *et al.*, 1994). Deproteinisation was done by a slightly modified method of Block *et al.* (1966). Samples were diluted ten times with cold distilled water and then 5% (w/v) sulfosalicyclic acid was mixed in 1:1 ratio followed by centrifugation at 9000g at 4°C. The free amino acids were investigated from the supernatant.

Assay method: The ion exchange chromatographic method was considered for the determination of free amino acids profile. The samples were analyzed in an automated amino acid analyzer (Pharmacia LKB Alpha Plus) at the Centre for Cellular and Molecular Biology, Hyderabad, India.

Statistical analysis: The data were pooled and mean value and standard deviation was calculated.

RESULTS

Comparison between the two larval instars: Seventeen free amino acids were identified in the haemolymph of fourth and fifth instar larvae, of which nine were essential, seven non-essential

Free amino acid in haemolymph of *Antheraea assama*Ww larvae

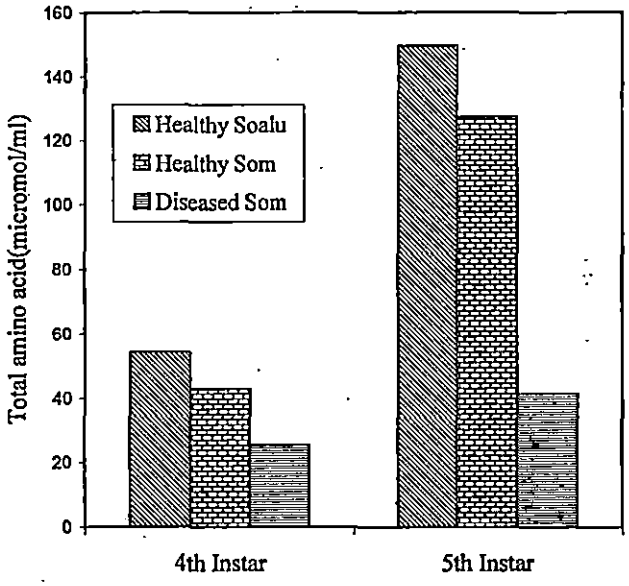

Fig. 1. Total free amino acid contents in the haemolymph of healthy and diseased (reared on som) larvae of *A. assama.*

and one semi-essential amino acid. The essential amino acids identified in the samples were threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine and arginine and non-essential amino acids were aspartic acid, serine, glutamic acid, glycine, alanine, cystine and tyrosine. The proline was the lone semi-essential amino acid as it is in case of *B. mori* (Bose *et al.,* 1989). The fifth instar larvae contained 127.1349 – 149.5567 µmol/ml of total free amino acids (Table I) in their haemolymph, which were approximately 2.86 times higher than in the corresponding fourth instar larvae. The total free amino acids in the haemolymph of fourth and fifth instar diseased worms were 25.3481 and 41.4271 µmol/ml respectively. The ratio of non-essential to essential amino acids was almost the same in both the instars of healthy worms (1.16 – 1.31). In diseased worm the ratio was 1.69 in fourth instar and 1.88 in fifth instar. In fourth instar healthy worm lysine was the major essential amino acid (19.97 – 21.54 per cent of the total) and glutamic acid was the major non-essential amino acids (17.47 – 19.45 per cent) (Figs. 2A and B). In the fifth instar lysine content was 10.06 – 16.68 per cent and acidic amino acids, aspartic acid and glutamic acid together were of very high (approximately 25 per cent) proportions (Figs. 3A and B).

Comparison of host plant (Som and Soalu) related differences: The qualitative variation of free amino acids was the same in both the fourth (Figs. 2A and B) and fifth (Figs. 3A and B) instar healthy larvae reared separately on som and soalu plant but there was a quantitative variation of the total free amino acids in the haemolymph of the larvae reared on the two plants (Table I) (Fig. 1). The larvae reared on soalu plant recorded a higher amount of free amino acids (54.4102 µmol/ml in fourth and 149.5567 µmol/ml in the fifth instar) in their haemolymph than in the larvae reared on som (42.9294 µmol/ml in fourth and 127.1349 µmol/ml in the fifth instar). Though there was a little difference in the percentage of different free amino acids in the fourth instar larvae reared on the two different host plants but it was almost similar in the fifth instar (Figs. 2A and 3A).

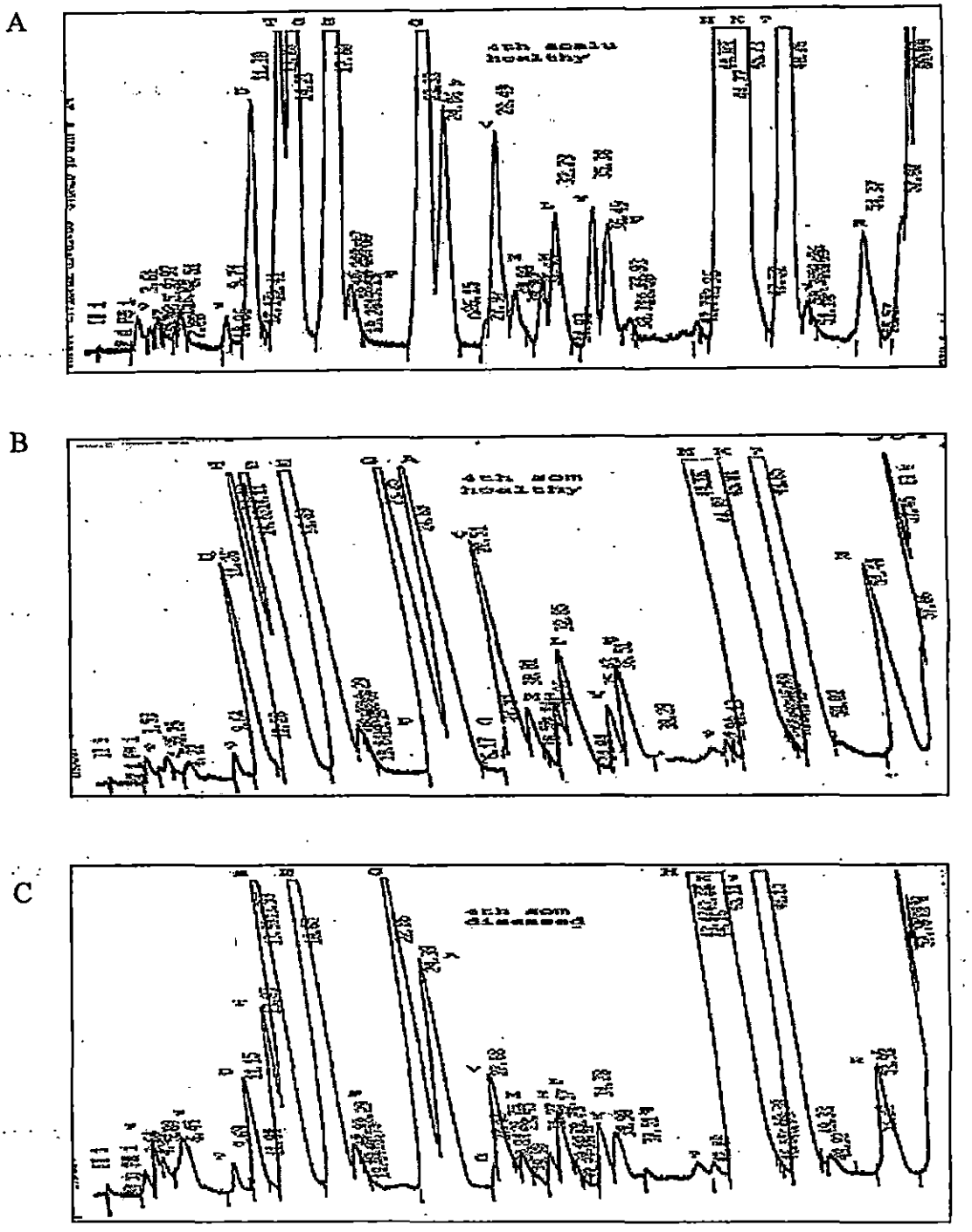

Fig. 2. Free amino acids profile in the haemolymph of fourth instar *A. assama*; (A) healthy larvae reared on soalu plant; (B) healthy larvae reared on som plant; (C) diseased larvae reared on som plant. [D=Aspartic acid, T=Threonine, S=Serine, E=Glutamic acid, P=Proline, G=Glycine, A=Alanine, C=Cystine, V=Valine, M=Methionine, I=Isoleucine, L=Leucine, Y=Tyrosine, F=Phenylalanine, H=Histidine, K=Lysine, R=Arginine, ?=unidentified].

Free amino acid in haemolymph of *Antheraea assama*Ww larvae

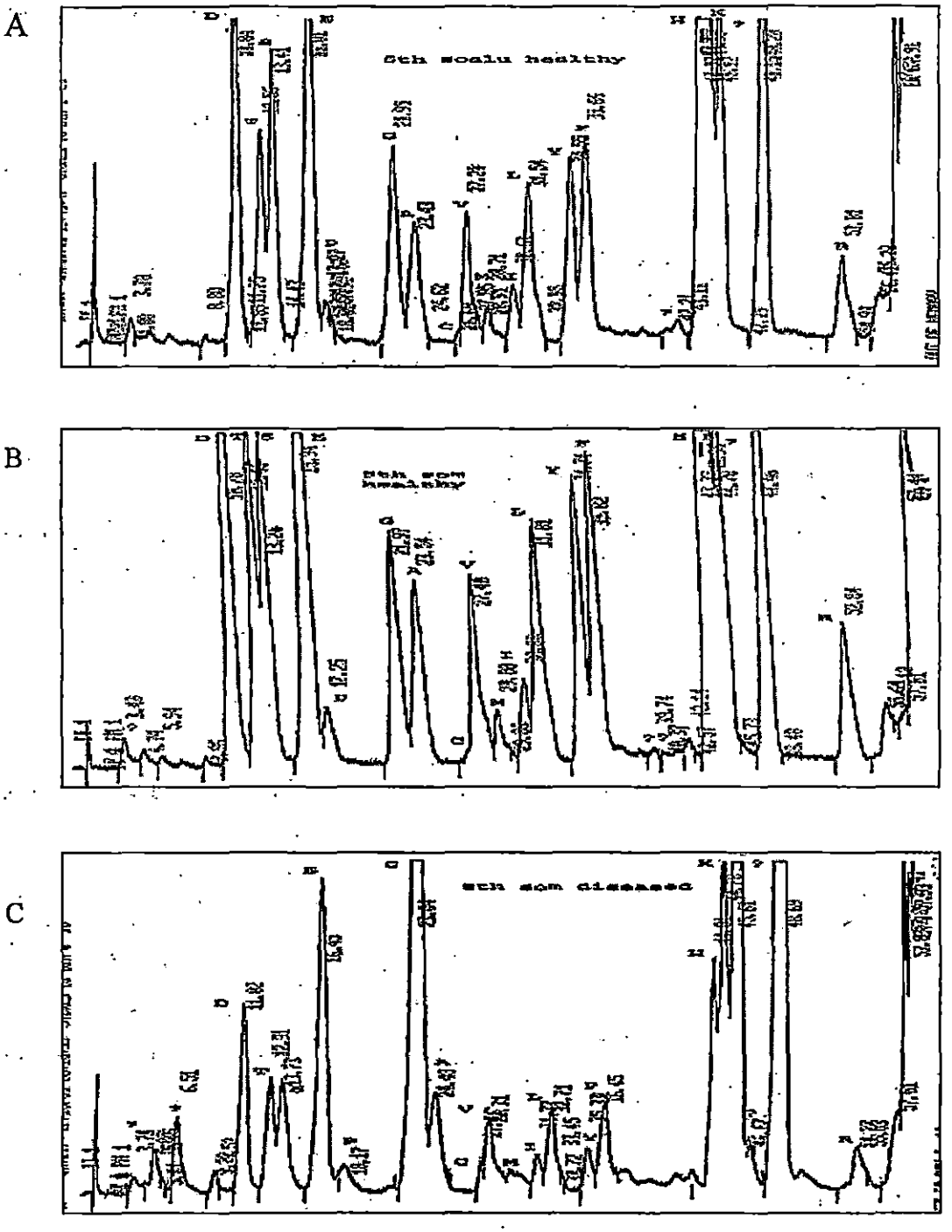

Fig. 3. Free amino acids profile in the haemolymph of fifth instar *A. assama*; (A) healthy larvae reared on soalu plant; (B) healthy larvae reared on som plant; (C) diseased larvae reared on som plant. [D=Aspartic acid; T=Threonine, S=Serine, E=Glutamic acid, P=Proline, G=Glycine, A=Alanine, C=Cystine, V=Valine, M=Methionine, I=Isoleucine, L=Leucine, Y=Tyrosine, F=Phenylalanine, H=Histidine, K=Lysine, R=Arginine, ?=unidentified].

Table I. Free amino acid (μmol/ml) profiles in the haemolymph of healthy (reared on soalu and som plants) and bacterial flacherie diseased (reared on som plant) larvae of *A. assama* (mean+SD).

Amino acid (μmol/ml)	Fourth instar			Fifth instar		
	Healthy		Diseased	Healthy		Diseased
	Soalu	Som	Som	Soalu	Som	Som
Aspartic acid(NE)	1.1824±0.27	1.3984±0.22	1.1189±0.23	15.6713±0.64	15.0533±1.45	2.4981±1.32
Serine(NE)	7.3986±0.35	4.5824±0.38	2.8365±0.04	10.8745±0.63	7.6085±0.67	1.6818±0.33
Glutamic acid(NE)	10.5812±0.54	7.5016±0.73	3.8449±0.31	21.1243±0.56	16.8576±0.64	3.7941±0.50
Proline(NE/SE)	2.8626±0.34	2.4162±0.26	1.1489±0.14	7.1716±0.75	12.2179±0.28	2.0072±0.73
Glycine(NE)	5.4416±0.58	3.5534±0.53	4.7123±0.58	9.8315±0.74	6.4023±0.89	15.1112±0.99
Alanine(NE)	1.9286±0.28	3.1574±0.21	1.8849±0.16	6.9274±0.53	6.1591±0.61	1.2382±0.73
Cysteine(NE)	0.7114±0.14	0.0666±0.03	0.0619±0.04	0.1733±0.41	0.3471±0.10	0.1400±0.26
Tyrosine(NE)	0.7766±0.12	0.3801±0.05	0.3342±0.08	8.8636±0.40	7.2355±0.28	0.5773±0.42
Threonine(E)	2.6039±0.51	2.5060±0.23	1.2242±0.19	8.8673±0.61	9.3179±0.68	1.6558±0.54
Valine(E)	1.3984±0.22	1.6767±0.15	0.7213±0.19	4.7399±0.55	4.7736±0.42	1.6529±0.07
Methionine(E)	0.3509±0.22	0.3663±0.11	0.2547±0.11	0.8033±0.36	0.6711±0.48	1.2562±0.06
Isoleucine(E)	0.4843±0.08	0.5132±0.07	0.2351±0.11	2.8228±0.59	2.5513±0.25	0.2698±0.25
Leucine(E)	0.8337±0.22	0.8156±0.14	0.3469±0.07	6.9711±0.66	6.3686±0.93	0.9561±0.51
Phenylalanine(E)	0.7846±0.13	0.7157±0.08	0.3119±0.20	12.2920±0.44	8.9327±0.30	1.9457±0.72
Histidine(E)	3.5136±0.35	3.0141±0.20	1.2795±0.14	13.1819±0.51	8.4187±0.60	2.3253±0.98
Lysine(E)	11.7181±0.49	8.5737±0.52	4.2292±0.12	15.0430±0.78	10.0505±1.07	3.5973±0.33
Arginine(E)	1.8397±0.16	1.6960±0.20	0.8028±0.22	4.1983±0.48	4.1692±0.49	0.7202±0.32
Nonessential	56.76%	53.70%	62.89%	53.92%	56.54%	65.29%
Essential	43.24%	46.30%	37.11%	46.08%	43.86%	34.71%
Total	**54.4102**	**42.9294**	**25.3481**	**149.5567**	**127.1349**	**41.4271**

E= Essential, NE= Non-essential, SD= Standard Deviation.

Comparison between healthy and diseased worms: Diseased worms contained a total of 25.3481 µmol/ml free amino acid in the haemolymph of fourth instar and 41.4271 µmol/ml in the fifth instar. The values were 1.69 and 3.0 times less than their healthy counterparts respectively (Fig. 1). Glycine was the major amino acid (18.59 per cent in fourth and 36.48 per cent in fifth) in both the instars of diseased worms (Figs. 2C and 3C). All the amino acids except glycine were of very low quantity than in the healthy worms. The quantity of glycine was even higher (4.7123 µmol/ml in fourth and 15.1112 µmol/ml in fifth) than in the healthy larvae (3.5534 µmol/ml in fourth and 6.4023 µmol/ml in fifth). The ratio of non-essential to essential amino acids was higher (1.69 in fourth and 1.88 in fifth) in diseased worms than their healthy counterparts (1.16 in healthy fourth and 1.30 in healthy fifth). Though healthy larvae showed approximately 2.96 times increase in total free amino acid content in the fifth instar than in the fourth, the increase was only 1.63 times in the diseased larvae (Table I). There was a 5.98 times increase in total aromatic amino acids (histidine, phenylalanine and tyrosine) in the fifth instar healthy larvae (4.1099 µmol/ml in fourth and 24.5869 µmol/ml in fifth) but in the diseased larvae it was only 2.52 times (1.9256 µmol/ml in fourth and 4.8483 µmol/ml in fifth). Acidic amino acids such as aspartic acid and glutamic acid increased 3:59 times in healthy (8.896 µmol/ml in fourth and 31.9108 µmol/ml in fifth) and 1.27 times in diseased (4.9638 µmol/ml in fourth and 6.2922 µmol/ml in fifth) larvae. Basic amino acids such as histidine, lysine and arginine increased 1.7 times in healthy (13.2838 µmol/ml in fourth and 22.6384 µmol/ml in fifth) while 1.05 times in diseased (6.3115 µmol/ml in fourth and 6.6428 µmol/ml in fifth) larvae.

DISCUSSION

The increase of the concentration of total free amino acid in the haemolymph of healthy *A. assama* corroborates with the findings of Das *et al.* 2004. The amino acids obtained by Sharma *et al.* (1995) and in this study were almost the same except the amine form of glutamic acid and aspartic acid obtained in the earlier observation and proline and cysteine in our observation. Furthermore, Sharma *et al.* (1995) did not obtained glutamic acid, proline and the sulphur containing amino acid cysteine in the fourth stage and proline and cysteine in the fifth stage larvae. There was no earlier record of proline and cysteine in *A. assama.* Proline is very essential for the proper growth of the larvae (Inokuchi, 1969; Bose *et al.,* 1989). Cysteine is important in intermediary metabolism and serves as a source of sulphhydryl group for the synthesis of coenzymes and hormones (Gilmour, 1961). In *A. assama* it was recorded that the nonessential amino acids dominated the essential amino acids like in other arthropods (Claybrook, 1983; Sharma *et al.,* 1995). According to Dhavalikar (1962a, b) there were seventeen amino acids present in the muga silk filament. Sixteen amino acids are common with our observation but we could not detect tryptophan. This might be due to the absence of tryptophan in the leaves of both the som and soalu (Sinha and Sinha, 1991). Tryptophan is a component of muga silk filament. *A. assama* may have the ability to convert indole acetic acid to tryptophan likewise in *B. mori* larvae (Kikkawa, 1941). The larvae fed with soalu leaves showed a higher amount of amino acids than the larvae fed on som leaves. This corroborates the presence of a higher amount of amino acids in the soalu leaves (Sinha and Sinha, 1991). The unidentified peaks found in all the samples might be the derivatives of amino acids, which require a thorough study for their identification and significance.

Incidence of disease was more frequent in the fifth instar stage (Das *et al.*, 2006). Hence in this instar the diseased worms had 3.0 times less amino acid content in their haemolymph than in their

healthy counterparts. A lower content of amino acids in the diseased worms might be due to an interference of the pathogen in the larval physiology (Sinha *et al.*, 1988b). Chitra et al. (1974), Kodama and Nakasuji (1968) have reported a fall in the gut pH, due to bacterial infections. This may impair absorption process in gut. The acidic amino acids, aspartic acid and glutamic acid were drastically reduced in the diseased worms, which might be responsible for the retarded growth of the diseased worms (Arai, 1977; Ito and Arai, 1966). Both aspartate and glutamate play active role in amino acid nutrition as donors of amino groups in the transamination reactions (Bheemeswar and Sreenivasaya, 1952). The basic amino acids, arginine, lysine and histidine are involved in the regulation of haemolymph osmotic pressure (Florkin and Jeuniaux, 1974). These were obtained in a very low quantity in the diseased worms than in their healthy counterparts. Glycine plays an important role in detoxication mechanism (Fredler and Smith, 1954; Shyamala, 1964). Our investigation records a higher concentration (1.33 – 2.36 times) of glycine in diseased worms when compared with that of healthy larvae. This warrants further investigation to find out the actual role of glycine in diseased worms.

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Haemolymph Protein Profiles of Healthy and Bacteriosis Affected Larvae of *Antheraea assama* Ww (Lepidoptera:Saturniidae)

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ABSTRACT

A comparative quantitative study of haemolymph protein profile was undertaken in the larvae of *Antheraea assama* Ww (Lepidoptera:Saturniidae). Fifth instar healthy and the larvae suffering from bacteriosis were considered for the study. The study was undertaken in the non-traditional district of Coochbehar, WB, where attempt has been made to rear this silkmoth on commercial basis since 1986. Irrespective of stages the females contained higher protein content than the males. Seasonal variations of haemolymph total protein content were also recorded. The HPLC assay revealed 10-11 protein peaks in the healthy as well as in diseased fifth instar worms. But by SDS-PAGE revealed 14-15 peptide bands in the same larvae. Larvae suffering from bacterial toxicosis had 16 peptide bands in its haemolymph.

Key words: *Antheraea assama,* haemolymph protein profile, bacteriosis.

INTRODUCTION

The precious muga silk is obtained from the silkmoth *Antheraea assama*Ww (Lepidoptera: Saturniidae). The species is endemic to Assam and Meghalaya. A venture has been taken for commercial rearing of the species in the district of Coochbehar, West Bengal, India, a non-traditional area. Despite immense potentiality of muga culture in this non-traditional area, its productivity in terms of cocoon yield per laying is far from satisfactory. One of the major causes of low production is the high incidence of diseases such as bacteriosis.

The occurrence of bacteriosis is associated with the unevenness of environmental factors like temperature and relative humidity (Chishti *et al.*, 1991). Kakati (1991) recorded a 34.82 percent of annual mortality of muga silkworms due to diseases in North-East region of India of which flacherie was recorded maximum (16.38 percent). On an average 11 – 15 per cent mortality per year due to bacteriosis was recorded in the Coochbehar district; the highest incidence (24.97 percent) was recorded in 'Bhodia' (Aug.-Sept.) crop (Das *et al.*, 2006). Bacteria belonging to the Genera *Pseudomonas, Klebsiella, Citrobactor, Proteus, Providentia and Bacillus* were identified from bacteriosis affected muga silkworm in this district (Das *et al.*, 2006).

The growth, quantity and quality of silk produced by the silkworms and their fecundity depends largely on available nutrients in leaves and the efficiency of conversion of leave nutrients into biomass (Chaluvaachari and Bongale, 1996; Kar *et al.*, 1994; Sarkar *et al.*, 1992; Sinha *et al.*, 1993 and Ray *et al.*, 2001). The most valuable nutrient is the protein content which has

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significant role on all the metabolic activities such as growth, metamorphosis and fecundity (Kar *et al.*, 1994). Quite an extensive research was done on the haemolymph protein content of the mulberry silkworm, *Bombyx mori* (Lauffer, 1943; Watt and Pan, 1978; Ogawa and Tajo, 1981). In *A. assama* only a few preliminary works on haemolymph protein content have been done. Depali Devi (1993) estimated the protein content of healthy larvae in relation to the various environmental factors; Chetia and Handique (1997) studied the impact of automobile fumes on the protein content and Das and Deb (2005) assayed the protein content of healthy larvae reared on two major host plants and their impact on cocoon characters. A sharp depletion of major free amino acid contents in the haemolymph of muga silkworm having bacteriosis was also observed by Das and Deb (2007).

In order to evaluate the performance of the species in this non-traditional area and to have a better understanding of intricacies of changes in the level of haemolymph protein, a detail study was carried out on the haemolymph protein profile of healthy and diseased fifth stage muga silkworms. Moreover the differences in the protein / peptide profiles between the healthy and diseased fifth instar female larvae, which largely contribute to the economy, were also assayed.

MATERIALS AND METHODS

Larval source: Healthy fourth and fifth instar larvae of *A. assama* immediately after gut purging were collected from the Muga Basic Seed Farm, Khagrabari, Directorate of Sericulture, Government of West Bengal. Diseased worms were collected from the fields of registered farmers of different subdivisions of Coochbehar district. In both the cases the larvae were reared on som plant (*Persea* bombycina). Worms suffering from bacteriosis were identified by their clinical symptoms based on (Chishti *et al.*, 1991; Sanakal *et al.*, 1996; Thangavalu *et al.*, 1988; Govindan and Devaiah, 1995 and Das *et al.*, 2006). The worms were always collected at dawn. Two-day-old fifth instar healthy and diseased (early stage) larvae were considered for the haemolymph analysis by HPLC and SDS-PAGE, because the symptoms of the disease were clear and the protein contents were highest in the diseased worm on that day. The study was undertaken on the Bhodia season (Aug-Sept), for the incidence of bacteriosis was highest during this season.

Haemolymph collection: The abdominal legs of healthy and diseased larvae were pricked with glass capillary tube and haemolymph was collected in ice-cold Eppendorf tubes. A pinch of phenylthiourea and EDTA was added to the sample to avoid melanisation (Cheung *et al.*, 1978) and clotting (Wheeler, 1963).

Assay methods: Samples after centrifugation at 3000g for 2 minutes (Kar *et al.*, 1994) were subjected to protein estimation by Lowry's method (Lowry *et al.*, 1951). Absorbances were taken by using semi-autoanalyser (ERBA Chem Pro-5; Transasia Biomedicals LTD.). All tests were repeated thrice.

The comparison of protein / peptide contents of healthy worms and those suffering from bacteriosis were evaluated by reverse phase HPLC. The samples were analyzed at the Centre for Cellular and Molecular Biology, Hyderabad, India.

The molecular weight of the peptides present in the haemolymph of healthy and bacteriosis affected larvae were determined by SDS-PAGE (Laemmeli, 1970) and with the help of a computer aided Vilber Lourmat (France) Gel Document System and software Bio1D.

Statistical analysis: The data of three years were pooled and mean values and standard errors were calculated The CD value was determined using ANOVA (Indostat).

RESULTS

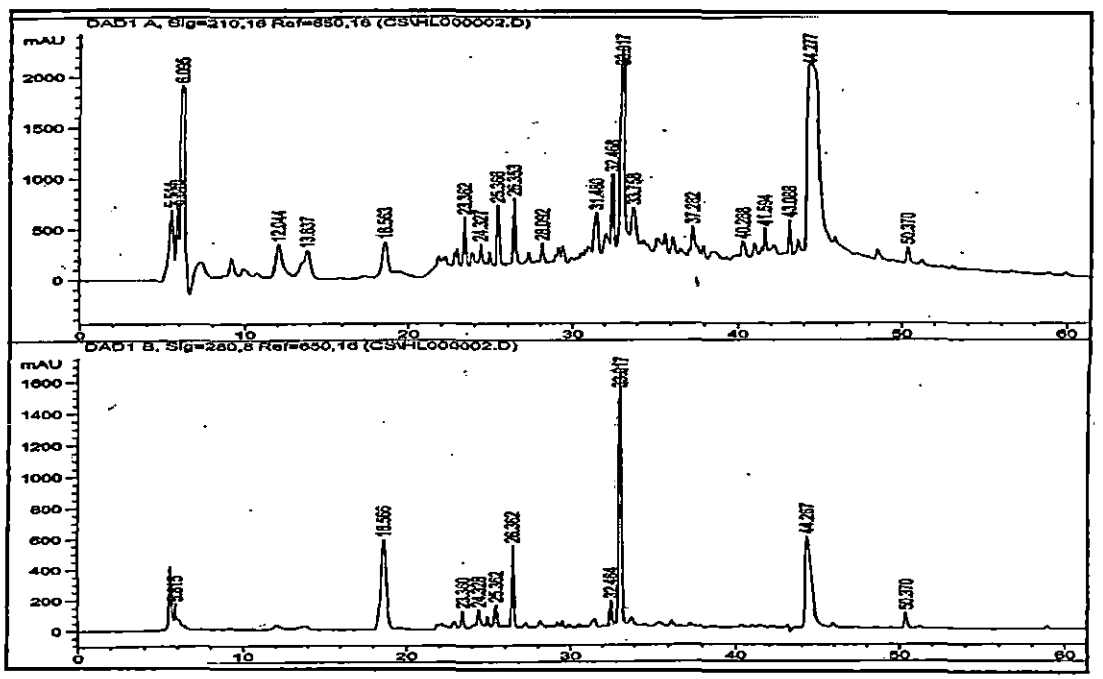
Haemolymph protein contents of healthy and diseased worms: It was found that during Bhodia season (Aug-Sept), the clear symptoms of disease in larvae appeared from the third day of the fifth instar and they died variably within sixth day with some exceptions where the symptoms appeared on fifth day and the larvae died on seventh or eighth day, but the healthy larvae ripened after another three days and started cocooning. It was observed that in healthy larvae protein content quantitatively increased significantly with the progress of days attaining peak on the ninth day (40.56 mg/ml), but in case of diseased larvae the peak protein content was recorded in 2-day-old larvae (16.52 mg/ml), then decreased significantly, the lowest was being on the fifth day (4.40 mg/ml), after which the larvae died. In the first day of appearance of the symptoms there was no significant difference in the protein content between diseased (15.34 mg/ml) and healthy (16.02 mg/ml) larvae. But afterwards, in the diseased larvae the content decreased significantly in the subsequent days with an exception of a slight increase on the second day (16.52 mg/ml) (Table-I). In the day before the last (usually on the fourth day) the amount (9.04 mg/ml) decreased more than two times that of the healthy (22.26 mg/ml), and in the last day (usually on sixth day) the amount (4.40 mg/ml) decreased seven times than that of the healthy one (Table-I). In contrast, in healthy larvae the haemolymph protein content increased rapidly from fourth day onwards and on the final day it was almost double the amount of the fourth day.

TableI: Day wise variation of haemolymph total protein (mg/ml) content (Mean±SE) in the 5th instar healthy and bacteriosis affected muga worms during Bhodia season.

Day	Haemolymph protein (mg/ml)	
	Healthy	**Bacteriosis**
1st	16.02 (±1.60)	15.34 (±1.54)
2nd	16.10 (±1.31)	16.52 (±1.46)
3rd	15.86 (±2.62)	11.21 (±1.34)
4th	22.26 (±3.04)	9.04 (±1.02)
5th	29.40 (±2.52)	4.40 (±0.58)
6th	31.28 (±2.84)	Death of larvae
7th	35.24 (±2.49)	——
8th	37.42 (±3.17)	——
9th	40.56 (±4.40)	——
CD(P=0.05):	Day= 1.76;	Larvae= 1.19
	Day X Larvae= 2.26	

Qualitative haemolymph protein contents of diseased and healthy worms: Haemolymph of fifth instar, both male and female, contained the major peaks at around 18, 33 and at 44 minutes. The fifth instar healthy male and female worms had a total of ten protein/peptide peaks in their haemolymph (Table-II; Fig-I). The five protein / peptide peaks appeared in the assay at around 5,17-18, 32-33, 44 and 50 minutes in most of the haemolymph samples, but

(a)



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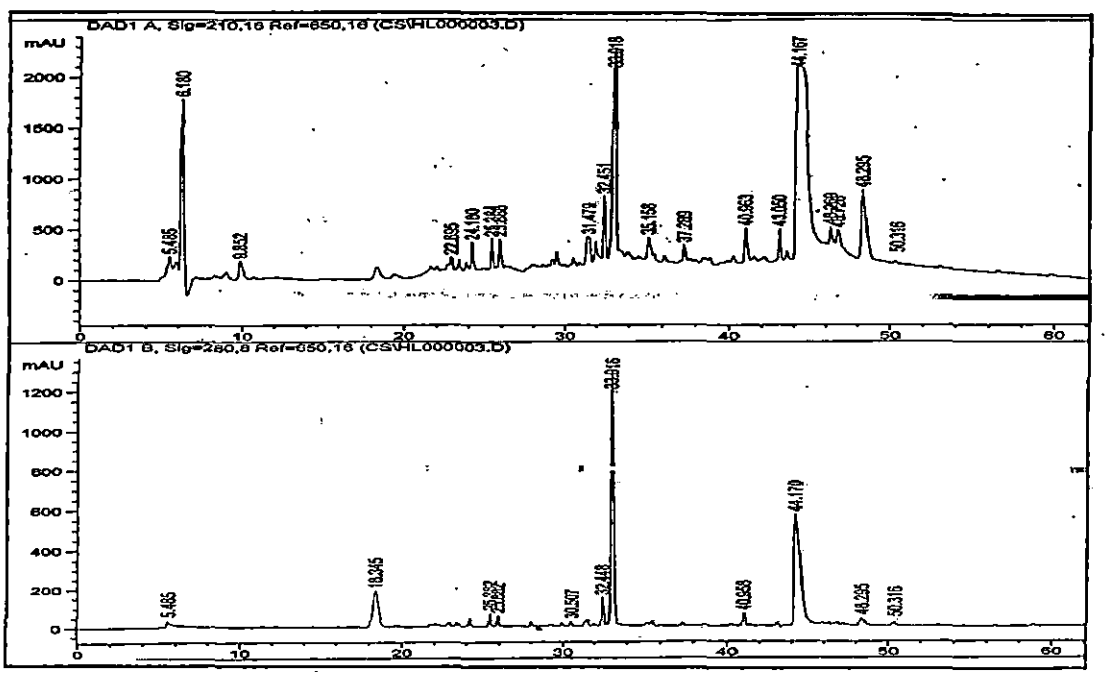
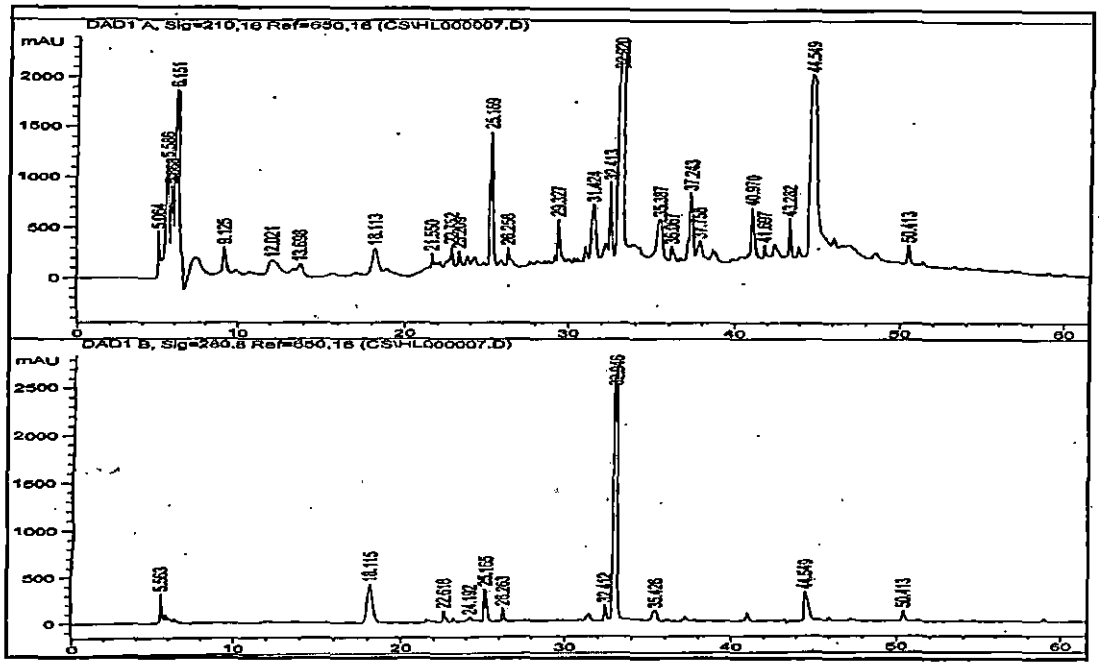


Fig.1: Reverse phase HPLC chromatogram of the haemolymph proteins / peptides of healthy fifth stage larvae of *A. assama.* (a) Male, (b) Female.

Haemolymph protein content of *A. assama*

(a)



(b)

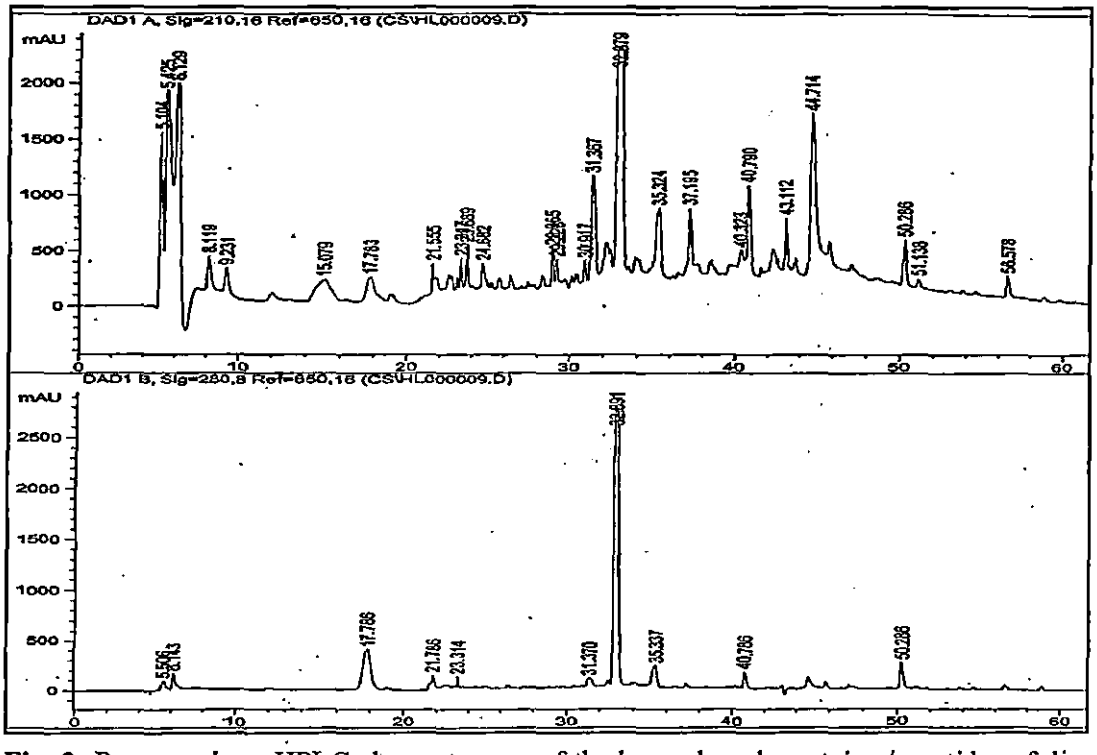


Fig.-2: Reverse phase HPLC chromatogram of the haemolymph proteins / peptides of diseased fifth instar larvae of *A. assama.* (a) At early stage of bacteriosis, (b) At late stage of bacteriosis.

the protein peak at around 50 min did not appear in healthy female and the other around 44 min did not appear in female larvae at late bacteriosis stage. Ten to eleven protein peptide peaks were recorded in the haemolymph of the worms at different stages of bactriosis such as early and late bacteriosis and bacterial toxicosis (Table II, Fig, 2, 3).

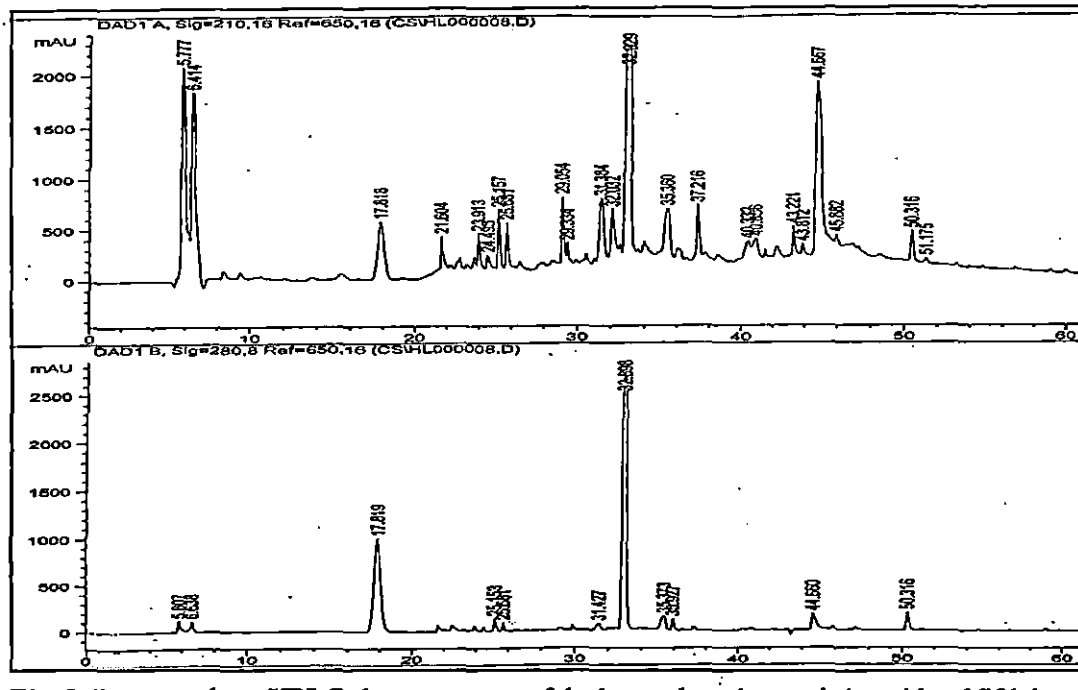


Fig. 3: Reverse phase HPLC chromatogram of the haemolymph protein/peptide of fifth instar female at bacterial toxicosis stage of *A. assama.*

Table II: Elution time (in minute) of haemolymph protein / peptide profile of Vth instar healthy and diseased muga silkworm assayed by HPLC

	Healthy worms		Diseased		
	Male	Female	At early stage	At late stage	At toxicos is stage
1	5.815	5.485	5.563	5.506	5.807
2	18.566	18.345	18.115	6.143	6.638
3	23.360	25.382	22.618	17.786	17.819
4	24.328	25.882	24.192	21.786	25.153
5	25.362	30.507	25.165	23.314	25.681
6	26.362	32.448	26.263	31.370	31.427
7	32.464	33.016	32.412	32.891	32.898
8	33.017	40.958	32.946	35.337	35.373
9	44.267	44.170	35.426	40.786	35.927
10	50.370	48.295	44.549	50.286	44.660
11	—	—	50.413	—	50.316

Table III: Molecular weight (Kda) of the haemolymph peptides of fifth instar healthy and diseased muga silkworm assayed by SDS-PAGE. Lane 1: Protein maker.

Lane:	6	7	8	9	10
Sl. No.	Healthy worm		Diseased worm (female)		
	Female	Male	At toxicosis stage	At late stage	At early stage
1	112.161	108.336	116.336	116.000	119.320
2	100.782	92.043	89.905	86.455	93.452
3	76.829	74.544	66.200	71.153	66.200
4	56.911	59.564	58.238	56.911	56.911
5	36.126	42.358	45.000	47.642	46.321
6	32.525	33.284	37.301	36.126	36.126
7	31.770	31.019	33.284	33.094	33.094
8	30.460	30.300	32.525	31.770	31.206
9	29.172	28.628	30.833	27.030	29.721
10	28.089	26.169	28.628	25.495	27.557
11	26.340	24.194	27.030	24.036	26.340
12	25.495	20.741	25.000	20.847	24.675
13	23.568	18.104	23.879	19.033	18.300
14	18.104	17.465	20.717	17.915	17.731
15	17.465	—	17.822	17.130	17.049
16	—	—	17.295	—	—

The electropherogram of the SDS-PAGE assay is presented in Fig. 4. Fifteen and fourteen peptide bands were recorded from the haemolymph of healthy female and male worms respectively. Out of 15, females had the major peptides with molecular weights of 112, 56,32, 26, 23 18 and 17 Kda and males had 108, 42, 33, 24, 18 and 17 Kda (Table-III). The diseased female worms of both early and late stages had a total of fifteen bands of which at early stage showed the major bands of 119, 56, 46, 33, 27, 24, 18 and 17 Kda and late stage showed the major peptides of 116, 71, 33, 27, 24, 20 and 17 Kda. Fifth instar female worms suffering from bacterial toxicosis exhibited a total of 16 peptide bands, of which five were major having the molecular weights 116, 58, 28, 20 and 17 Kda.

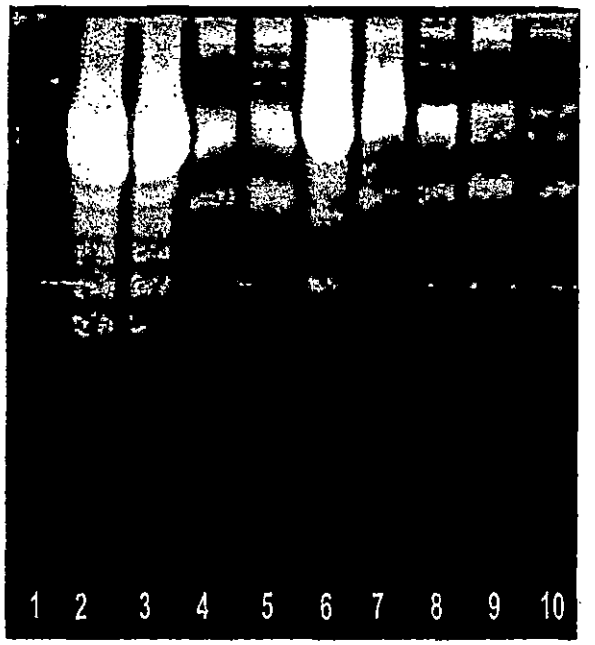


Fig.-4: Electropherogram (SDS-PAGE) of haemolympt peptides of the fifth instar larvae of *A. assama.*

DISCUSSION

The quantity of total haemolymph protein has been almost equal in diseased and healthy larvae at the onset of disease but it depletes rapidly in diseased worms, and abnormally falls too low just before their death. The sharp decline may be due to progressive impairment of the intestinal function of the diseased worms affecting the digestion and absorption of food, thereby decreasing the total quantity of protein in haemolymph (Gururaj *et al.*, 1999). A slight increase in the protein content of 48 hr day-old larvae might be due to initial increase of the metabolic rate of diseased worms for resisting the severity of infection. The rapid increase of protein content in the healthy fifth instar larvae is associated with the accumulation of storage pool for oocyte growth and silk protein. (Sinha *et. al.*, 1992; and Bashamohideen and Ameen, 1998).

The protein / peptide peaks appeared at 17-18 min. and at 32-33 minutes are the major constituents of haemolymph protein of all the samples. The peaks appeared at around 5 min and 18 minutes are the most polar regarding solubility, those appeared around 44 min and 50 minutes are the least polar (might be non-polar), and the major peak appeared at 32-33 min may be considered comparatively neutral protein constituent of haemolymph. One of the major peak of healthy fifth instar appeared around 44 min sharply goes down in all worms suffering from bacteriosis which requires further investigation to find out its role. One protein / peptide peak appeared in worms at early bacteriosis and at bacterial toxicosis (though the appearance time is different) needs further investigation to explain its role and immunological significance, if any, in the diseased worms. The appearance and disappearance of some of the peaks in diseased worms compared to those of healthy ones also need further investigation. The SDS-PAGE assay of the haemolymph samples reveals that all the healthy worms have irrespective of their sexes, five major peptides of 108-112, 32-33, 28, 18, and 17 Kda,. The major bands appeared in fifth instar healthy female worms are of 56, 26 and 23 Kda. These have not been appeared in the corresponding males. These peptides might be considered as female specific and the bands appeared at 42 and 24 Kda in healthy fifth instar male might be considered as male specific peptides. The major peptides having molecular weights of 32-33 and 17 Kda were present in all the bacteriosis affected worms as well the healthy worms. Therefore these can be considered as essential peptides of muga silkworm having general physiological significance. All bacteriosis affected worms have expressed peptides in the range of 116-119 Kda, which are heavier than those peptides in the healthy worms. The number of protein / peptide bands recorded by Depali Devi (1993) in the fourth (16 in Jarua season and 19 in other rearing seasons) and in the fifth (19 in Jarua and 21 in other seasons) instars are not similar to the present observation. The differences warrant for further study regarding changes in the number of peptide bands in haemolymph particularly with respect to seasonal environmental impact, if any. The reduced band width / peak of the protein / peptides of the haemolymph of the diseased worms might be due to the proteases released by the infected bacteria (Choudhury, *et al.* 2005); and the change of band pattern in these worms also require a thorough scrutiny. 23 Kda protein recorded by the other researchers (Choudhury, *et al.*, 2004; Kumari, *et al.*, 2005a, b; Unni, *et al.*, 2006) is more or less similar to 23-24 Kda peptides obtained in our investigation. Thus this preliminary observation on haemolymph proteins of A *assama* gives a future direction of research and to find out immunological linkage of the new peptides, if there be any.

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