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Antibacterial proteins from non-mulberry silkworms against flacherie causing *Pseudomonas aeruginosa* AC-3

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The muga silkworm, *Antheraea assama* (Ww) which produces golden yellow silk is indigenous to NE India, due to outdoor rearing it is susceptible to bacterial, viral and fungal infections. Diseases which are associated with pathogenic bacteria comes under the general term 'flacherie', which refers to the flaccid condition exhibited by silkworm due to different ailments and is caused by *Pseudomonas aeruginosa* AC-3. This communication reports the induction of antibacterial proteins in haemolymph of silkworm by injecting live non-pathogenic strain of *Pseudomonas* DAS-01. Protein profile of control and induced pupa were compared. In induced pupa 3 protein/peptide bands were found in low molecular weight region (18–24 kDa). These proteins/peptides were gel eluted as well as purified by Sephadex G-75 and were desalted by Sephadex G-25, G-10. The fractions were lyophilized and tested for antibacterial activity by both *in vitro* and *in vivo* methods. The fraction containing low molecular weight proteins were found to be effective in inhibiting the growth of *Pseudomonas aeruginosa* AC-3.

THE Muga silkworm, *Antheraea assama* is restricted to Northeast India, particularly Assam. These silkworms are reared outdoors, and hence are susceptible to many kinds of infections, i.e. bacterial, viral, fungal, etc. Among these, bacterial infection, collectively called 'flacherie', accounts for major loss of silkworms (Figure 1). It was found that these worms are naturally resistant to some strains of bacteria. This implies that there are some proteins present in the worms that are bactericidal in nature. If we can succeed in isolating and purifying the proteins and getting their amino acid sequenced, then it is possible to synthesize such proteins for further studies.

The defensive arsenal of insects, like that of man contains both passive structural barriers against infection and a cascade of active responses to organisms that gain access to the haemocoel following injury to integument. The primary defence of insects against pathogens and endoparasite is the prevention of infection via structural barriers such as rigid cuticle and peritrophic membrane that protects the mid gut. Even after this if the bacteria persist in the system, then initial haemolymph response is mediated by circulating haemocytes by the process of phagocytosis. If this innate mechanism of wiping out the antigen fails, synthesis of several proteins occurs, including lysozymes and other bactericidal proteins. The synthesis of these haemolymph proteins requires de novo RNA synthesis and follows a lag of minimum 8 h.

Muga silkworm is selected as a model system for studying humoral immunity, because it is of great economic importance due to its unique golden yellow silk and is the rarest variety of silkworm found exclusively in Northeast India. Pupal stage of this silkworm is selected for the experiments; the main advantage is that in the pupal stage the metabolic rate of silkworms is low, and injection with bacteria either live or attenuated, allows selective activation of genes for antibacterial protein synthesis. Potent antibacterial activity which appears in the haemolymph is attributed to low molecular weight proteins/peptides. In muga silkworm, causal organism[1] for 'flacherie' is *Pseudomonas aeruginosa*

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Figure 1. Healthy and diseased larvae of muga silkworm, *Antherea assama.*

AC-3. This study was undertaken to initiate *in vivo* synthesis of antibacterial proteins by induction and further isolation, purification and characterization of these proteins for checking their potential in inhibiting growth of pathogenic *Pseudomonas* strain.

Apart from lysozymes, an important part of the insect's immune system is the 'cecropins', a family of small basic proteins with potent antibacterial activity[2]. A similar antibacterial protein may be present in muga silkworm, which on further purification and characterization may play an important role in controlling the incidence of 'flacherie' in the worms.

Eggs of *A. assama* and *Philosamia ricini* were collected from Muga and Eri Training and Research Institute, Lahdoigarh, and also from local farmers. Newly hatched larvae were reared on som and castor plants at the Regional Research Laboratory (RRL), Jorhat campus by conventional methods[3]. Muga silkworms of 5–6-day-old pupal stage, weighing approximately 2 g were used for all experiments.

Pupae were divided into two sets, each containing 30–40 numbers in a group. One set was injected with sterile saline and the other with non-pathogenic *Pseudomonas* DAS-01 bacterial strain. Pupae were inoculated by injecting 2 μl of overnight grown culture of *Pseudomonas* DAS-01, described below in detail[4].

Pseudomonas DAS-01 was grown overnight in Luria Broth (g/l: tryptone 10, yeast extract 5, NaCl 5) in a rotory shaker (200 rpm, 30°C, Clime-O-Shake). The overnight grown culture was centrifuged the next day at 4°C and 5000 rpm, and the pellet was resuspended in sterile saline. Before injecting *A. assama* with the above strain, the number of *Pseudomonas* cells was counted by serial dilution method and was found to be 2×10^8 cells/ml. A dose of 2 μl of the above suspension was injected on the dorsal segment of the pupae by Hamilton micro syringe. For control at the same position, 2 μl Ringer's solution was injected.

Haemolymph was collected at an interval of one day from second till seventh day in autoclaved, prechilled eppendorff, and rinsed with 3% PTU (phenyl thio urea) under laminar flow. The pupae were given a slit under the dorsal region by a scalpel and haemolymph was collected and centrifuged immediately to get rid of haemocytes and stored at –45°C for further experiments.

Haemolymph collected from the control as well as bacteria-injected pupae were subjected to 15% SDS as well as native gel electrophoresis[5]. Haemolymph samples were diluted with sample buffer containing 5% mercaptoethanol and heated at 100°C for 3 min before application to the gel. Marker proteins (molecular weight 65, 45, 24, 18 and 14 kDa) were also loaded in appropriate concentration.

After comparing the protein profile of both the samples, it was observed that haemolymph of injected pupae contains few proteins/peptides in the low molecular weight region (~ 20 kDa). These bands were eluted from the gel along with the marker protein (bovine serum albumin; BSA) by dipping the gel pieces of interest in Tris buffer (1 M, pH 8.8) at 30°C for 24 h in an autoclaved eppendorff. It was then centrifuged (10,000 rpm) and supernatant was tested for antibacterial activity by inhibition zone assay method (eluted BSA acted as control).

The effectiveness in inhibiting the growth of *P. aeruginosa* AC-3 was tested on *P. aeruginosa*-spread agar
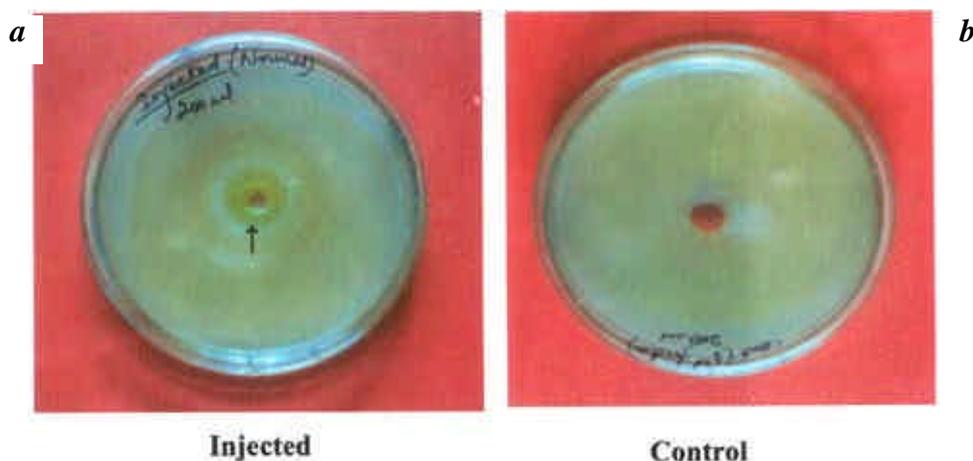
Figure 2. Antibacterial assay with pupal haemolymph injected with live bacteria and Ringer's solution (control).

plates by the above mentioned method. Low molecular weight proteins were found to be effective against them; the diameter of the inhibition zone was found to be 3.5 cm.

Further, the low molecular weight protein/peptide bands of haemolymph collected 2–7 days after injection were also eluted from the gel and tested for antibacterial activity. It was observed that the diameter of the zone was maximum in case of haemolymph collected four days after injection. Thereafter a decrease in diameter was observed, confirming a decline in antibacterial activity[2,6].

Estimation of haemolymph proteins was done by Folin–Ciocalteau's method. Haemolymph was collected after four days from pupa injected with non-pathogenic strain of *Pseudomonas* DAS-01 and also from pupa injected with Ringer's solution (acting as control). A known concentration of BSA was taken as control. In the injected sample protein concentration was found to be 16 mg/ml and in the control it was 15 mg/ml.

Haemolymph collected after four days from both bacteria-injected and control pupa was subjected to Sephadex column G-75. The column was equilibrated with Tris buffer (1 M, pH 8.8) and the haemplymph was diluted in the ratio 2 : 1 with the same buffer. Two peaks were observed and the eluents were lyophilized. Again to get rid of interfering salts from the fractions, it was desalted through Sephadex column G-25 and G-10. The fractions collected were lyophilized and tested for antibacterial activity.

After purifying the haemolymph through specific columns (Sephadex G-75, G-25, G-10), the fractions were collected and tested for antibacterial activity against *P. aeruginosa* AC-3 and *Pseudomonas* DAS-01 by inhibition zone assay method. The second fraction of bacteria-injected pupal haemolymph was found to be effective against both strains. To further confirm the results, all the fractions obtained from Sephadex column G-75 were lyophilized at –45°C and 500 µl of each fraction was added to 5 ml of Luria Bertini (LB) medium. To this, 100 µl of overnight grown *P. aeruginosa* AC-3 was added. Tubes were incubated at

30°C for 24 h. Growth was observed in all tubes, but was minimum in the tube containing the second fraction of bacteria-injected pupal haemolymph (obtained from column chromatography). OD was taken on a spectrophotometer at 600 nm, and was found to be minimum for the second fraction.

Two sets of experiments were performed *in vivo*. In the first set, 20–30 healthy muga larvae (late 5th instar) were taken and 10–20 of them were injected with 5 µl of the second fraction obtained from Sephdex G-75 column of bacteria-injected pupal haemolymph and ten were injected with 5 µl of the second fraction of saline-injected pupal haemolymph. After half an hour, they were challenged with 2 µl of overnight grown culture of *P. aeruginosa* AC-3. In the second set, a fresh batch of 20 muga larvae were taken (late 5th instar). Ten of them were injected with 5 µl *Pseudomonas* DAS-01 (non-pathogenic to silkworm) and ten were injected with 5 µl Ringer's solution. After three days the above 20 larvae were challenged with 2 µl *P. aeruginosa* AC-3 culture and fed with normal diet under ordinary conditions inside the insect cage.

Haemolymph collected from control as well as bacteria-injected pupa was tested for antibacterial activity by inhibition zone assay method and bacteria-injected pupal haemolymph was found to be effective against *P. aeruginosa* AC-3 (Figure 2). When the haemolymph was subjected to SDS and native gel electrophoresis, a group of three protein/peptide bands in the low molecular weight region was observed only in the injected pupa haemolymph (Figure 3). These bands were detected between 18 and 24 kDa molecular weight region.

These induced protein/peptide bands were gel-eluted and tested for antibacterial activity, and they showed positive results. In column chromatography with Sephadex G-75, two peaks were observed in both the injected as well as control pupal haemolymph. Fractions corresponding to the peaks 1 and 2 were collected and lyophilized. The lyophilized samples were tested for antibacterial activity. The

second fraction of bacteria-injected haemolymph was found to be effective against *P. aeruginosa* AC-3 and *Pseudomonas* DAS-01 itself (Figure 4). When these fractions were incubated with LB medium inoculated by *P. aeruginosa*

Figure 3. SDS–PAGE of pupal haemolymph injected with live bacteria and Ringer' solution showing antibacterial protein (shown by an arrow).

Figure 4. *a*, Fraction from column chromatography (Sephadex G-75). *b*, Antibacterial assay with fractions.

AC-3 for 24 h at 30°C, the same fraction was found to be effective against the above strains of bacteria, this was concluded after measuring their OD at 600 nm. It was minimum for the second fraction of injected haemolymph and thus growth was also minimum (Table 1). It was least turbid among all the test tubes.

The first set of larvae was immunized *in vivo* by injecting the second fraction collected from column G-75 and was challenged with pathogenic live strain of *Pseudomonas*. A control set injected with Ringer's solution was also maintained. Thirty per cent of the immunized set survived and started cocooning (Table 2 *a*; Figure 5).

In the second set , live non-pathogenic strain of *Pseudomonas* was injected and after 3–4 days challenged with pathogenic strain. Here, survival percentage was found to be 90 (Table 2). The low molecular proteins/peptides bands of haemolymph collected at one day interval from second to seventh day after injection were also gel-eluted and tested for antibacterial activity. It was observed that the diameter of the zone was maximum in case of haemolymph collected four days after injection (Figure 6).

In this study we report the induction of immunity in *A. assama* pupae by injection of live non-pathogenic strain of *Pseudomonas*. It has been known that insects inoculated with live non-pathogenic or killed bacteria can acquire resistance to subsequent challenge by bacterial pathogen[7]. Most experiments have used lepidopterans such as wax moth (*Galleria mellonella*) or silkworms for induction of immunity because of high volume of haemolymph in them. It was found that in *Drosophila*, response to microbial infections mounts a multifaceted immune response involving humoral reactions that culminate in destruction of invading organisms by lytic peptides[8]. In *Drosophila*, signalling pathways involved in fighting foreign elements were also established[9].

Extensive work on induced immunity in insects has been carried out[2,6,8–12]. It has been established that there are two stages to fight an infection. The first is removing the bacteria from circulation by phagocytosis and nodule formation[5]. The next step is *de novo* synthesis of RNA and specific proteins which give rise to increasing antibacterial activity in haemolymph. Studies on insect haemolymph revealed the presence of a variety of proteins in response to injury or bacterial challenge[13]. A total of 16 haemolymph proteins along with proteins associated with immunity were identified. As in our studies acrylamide gel is not a linear concentration gel and molecular markers
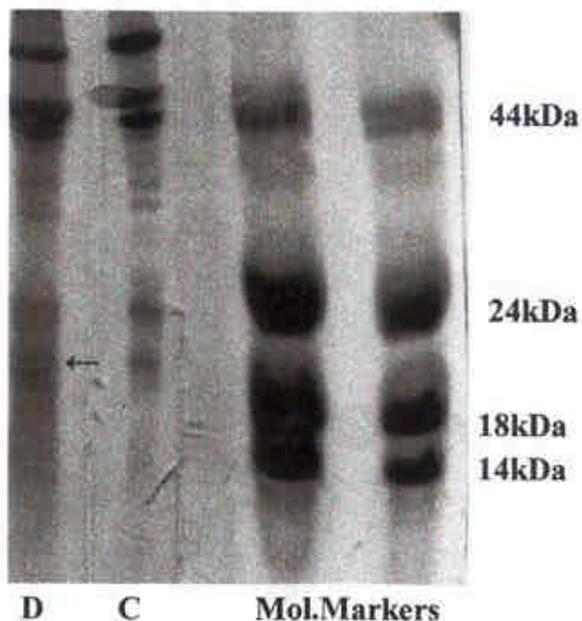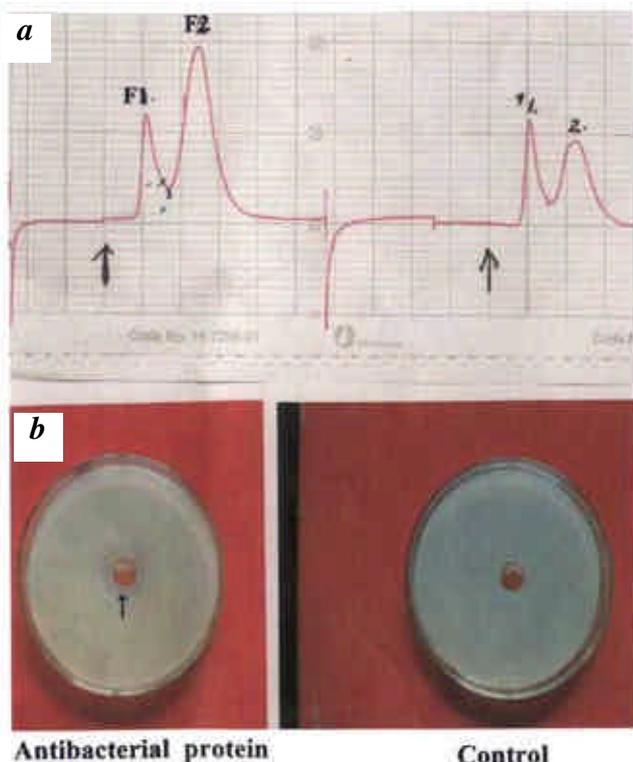
Table 1. Growth of *Pseudomonas aeruginosa* AC-3 in the medium

Fraction	Optical density taken at 600 nm
Fraction 1 (control)	0.73
Fraction 2 (control)	0.76
Fraction 1 (injected)	0.69
Fraction 2 (injected)	0.41

Diseased Larvae   Immunized Larvae

Figure 5. Immunized and control larvae of Eri silkworm, *Philosamia ricini.*

Table 2. *In vivo* experiments and immunization studies

| Time interval (in days)	Experimental set-up				Control set-up			
	Ten healthy larvae (initial)				Ten healthy larvae (initial)			
	No. of live larvae after 1, 2, 3, 4 days of injection				No. of live larvae after 1, 2, 3, 4 days of injection			
Haemolymph collected	1st expt	2nd expt	3rd expt	SD	1st expt	2nd expt	3rd expt	SD
First set								
After 1 day	5	6	6	± 0.46	2	4	4	± 0.81
After 2 days	4	4	5	± 0.32	1	3	2	± 0.81
After 3 days	3	4	4	± 0.46	0	2	0	± 0.92
After 4 days	3	4	4	± 0.46	0	0	0	± 0.00
Second set								
After 1 day	10	8	9	± 0.81	6	7	7	± 0.31
After 2 days	9	7	8	± 0.81	3	5	5	± 0.91
After 3 days	9	7	8	± 0.81	0	3	2	± 0.84
After 4 days	9	7	8	± 0.81	0	0	0	± 0.00

0, indicates all larvae are dead. SD, Standard deviation; expt, Experiment.

used were from 14 to 67 kDa, we could determine the weight of proteins occurring in this range. Sixteen bands were found in both injected and control pupa haemolymph, but a cluster of three protein/peptide bands was observed only in bacteria-injected haemolymph sample. These bands were inducible and are formed in response to bacterial challenge; they constitute the humoral defence line in muga silkworm. These were low molecular weight proteins and lie between 18 and 24 kDa. They are effective in fighting against Gram-negative bacteria such as *P. aeruginosa* AC-3, and *Pseudomonas* DAS-01, which was concluded after studying the antibacterial property of the isolated protein by inhibition zone assay method.

Humoral defence factors consists of phenoloxidases, lysozymes, lectins, haemolin, cecropins, attacins, etc.[14]. Phenoloxidase is a 80 kDa reactive protein which attacks the bacterial cell wall. Lysozyme, being a 11–28 kDa protein,

directly attacks bacteria by hydrolysing their peptidoglycan layer of the cell wall. Cecropins and attacins are small molecular weight proteins ranging from 4 to 10 kDa and are effective against both Gram-positive and Gram-negative bacteria. In general, the susceptibility of insects to pathogens seems to decrease as insects advance in age[15]. Induction of antibacterial activity as a function of time after immunization with live *Pseudomonas* also showed that (Figure 6) peak of immunity was on the fourth day after injection. Time taken to reach the peak of immunity varies among various insect species[16].

Haemolymph of insects contains many proteins; but after injecting the pupa with non pathogenic bacteria, along with normal haemolymph proteins which are constitutively present in them, some induced proteins are also formed. These were absent from Ringer's solution-injected control. Further protein estimation of haemolymph of both injected
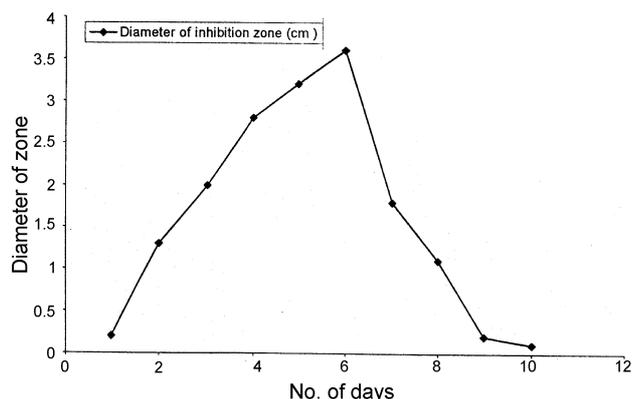
Figure 6. Relation between antibacterial protein activity and inhibition zone.

and control pupa was performed. After repeating the experiment three times with a new batch of pupae, it was found that protein concentration in injected pupa haemolymph was always higher by 1–1.5 mg/ml than its control counterpart. This difference can be attributed to induced proteins formed against bacteria for self-defence.

The fractions obtained from column chromatography with G-75 were again subjected to G-25 and G-10 column for desalting, as at each purification step we were losing some fraction of protein. Hence we repeated the procedure and the collected fractions were pooled together to get high concentration of protein. They were lyophilized and tested for antibacterial activity by both *in vivo* and *vitro* methods. Testing *in vitro* was done with inhibition zone assay and by incubating the fractions with overnight grown culture on *P. aeruginosa* AC-3. In both the tests, the second fraction obtained with injected pupal haemolymph was found to be effective against the bacteria. Lysozymes and some related antibacterial proteins are low molecular weight proteins[2]; thus the second fraction showing antibacterial activity is concordant with the established fact.

With *in vivo* experiments, we obtained further confirmation of our results. In one set of experiments, immunization was done with attenuated or heat-killed[17] strains of micro-organisms and antibodies are raised against them, whereas in another set, immunization was done directly with the isolated and purified antibacterial proteins. In insects, instead of antibodies we have antibacterial or bactericidal proteins, because even though these proteins fight the invader organism, structurally and functionally they are different from both humoral and cell-mediated antibodies formed in higher vertebrates. Here two sets of healthy larvae were immunized and challenged after specific time gap. In both the sets survival percentage was higher than that of respective control. The experiment was repeated thrice and survival percentage found to be similar. We can thus conclude that some inducible antibacterial proteins are present in muga silkworm, which requires

further purification and characterization. It is a complex multicomponent, inducible, haemolymph protein system involved in defence against bacterial infection.

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