Effect of Partially Purified Protease of *Pseudomonas aeruginosa* Strain AC-3 on *Antheraea assama* Westwood Larvae[1]

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Abstract The protease released by *Pseudomonas aeruginosa* strain AC-3, a causal organism of flacherie disease in *Antheraea assama* Westwood silkworms, was characterized and its activity against muga silkworm larvae was assessed in laboratory studies. When grown in casein broth maximum protease production occurred when the strain was cultivated for 60 h. This protease was partially purified by acetone precipitation and subjected to SDS-PAGE. Its molecular weight was approximately 35,000 da. The partially purified protease reduced larval survivability *in vivo*. The hemolymph protein profile revealed an apparent detrimental effect of the protease on biologically important proteins of silkworm larvae.

Key Words Protease, *Antheraea assama, Pseudomonas aeruginosa,* muga silkworm, silkworm, hemolymph proteins, flacherie disease

Diseases of silkworm larvae that are caused by pathogenic bacteria are generally referred to as "flacherie" due to the flaccid conditions exhibited by infected silkworms (Baig and Kumar 1987). The muga silkworm, *Antheraea assama* Westwood, that produces golden-yellow silk and is indigenous to the northeast India, routinely experiences a high incidence of flacherie. Entire broods may be eliminated in epizootics caused by the disease (Choudhury 1981).

Pseudomonas aeruginosa strain AC-3, isolated from diseased muga silkworm, was found to be one of the causal organisms of flacherie in this host (Guha et al. 1999, 2002). There are reports on the pathogenicity of *P. aeruginosa* (Schroeter) Migula on insects like adult grasshoppers, *Melanoplus bivattatus* Say and *Camnula pellucida* Scudder. *Pseudomonas aeruginosa* also produces lethal septicemia in the greater wax moth (*Galleria mellonella* L.) larvae, locusts, eastern tent caterpillar (*Melacosoma americanum* F.), and cutworms and hornworms (*Manduca* spp.) (Bulla 1975). Although reports are available on the infection of *Pseudomonas* sp. on silkworms (Krishnaswamy et al. 1973, Deviah 1994, Nagendran and Thiruvalluvan 1999), scientific data are lacking on the mechanism of pathogenesis of the bacterium on silkworm larvae. Therefore, in the present study, we report the effect of partially

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purified protease derived from *P. aeruginosa* strain AC-3 on silkworm larvae to characterize the pathogenesis of the bacterial strain in muga silkworm larvae.

Materials and Methods

Organisms. The *P. aeruginosa* strain AC-3 used in these studies was cultured on nutrient agar (NA) (Hi-Media Laboratories, Mumbai, India) slants. It was frequently subcultured and checked microscopically for purity.

Muga silkworm larvae used in these studies were derived from eggs collected from the Govt. Basic Seed Farm, Khanapara, Guwahati. Neonates were reared on som plants, *Persea bombycina* Kost., following conventional methods (Choudhury 1981). Fifth-instar larvae (2 to 3 days old) weighing an average of 2.75 g ± 0.30 were used for all the experiments.

Protease and proteolytic assays. *Pseudomonas aeruginosa* strain AC-3 was inoculated in a broth medium of 2.0% casein, 0.1% yeast extract, 0.1% $MgSO_4$, 0.1% $CaCl_2$, 0.001% $FeCl_2$ and 1.5% agar and incubated at 30°C on an orbital shaker at 200 rpm (Hoshino et al. 1997). The optimal incubation period for protease production was determined by removing samples of the broth at 12-h intervals for 72 h. Cells in these samples were extracted by centrifugation at 5,000 rpm for 10 min (Sorvall RC 5B Plus, Germany). The supernatant was treated with a reaction mixture for protease assay with incubation times of 0, 15, 30, 45, 60 and 75 min. The proteolytic activity within the supernatant was assayed as described by Hoshino et al. (1997) where 900 µl of 50mM phosphate buffer (pH 7.0) containing 0.5% casein was added to 100 µl of the supernatant. The reaction was stopped after 30 min of incubation at 30°C by the addition of 270 µl of 1M acetic acid-0.67 M sodium acetate solution containing 5.4% trichloroacetic acid. After an additional 30 min, the mixture was centrifuged, and the liberated proteolytic peptides were estimated by the method of Lowry et al. (1951). The experiments were repeated three times. One unit of protease activity was defined as the amount of the enzyme releasing 1 µg of tyrosine per min at 30°C. The specific activity is the number of enzyme units per mg of total protein.

A partially purified protease was obtained by 30% acetone precipitation of the supernatant and dissolving the residue in 0.05M phosphate buffer (pH 7.0). Protein was estimated by the method of Lowry et al. (1951). This protease mixture was further characterized with sodium dodecyl polycrylamide gel electrophoresis (SDS-PAGE). The basic methods employed were those of Laemelli (1970). The partially purified protease was diluted in SDS-sample buffer containing 5% mercaptoethanol and heated to 100°C for 3 min; 100 µg was loaded in the well. For determination of the molecular weight bovine serum albumin, 67 kd, egg albumin, 45 kd, trypsinogen, 24 kd, β-lactoglobulin, 20.1 kd and lysozyme 14.2 kd were used.

***In vivo* and *in vitro* protease activity.** Hemolymph was collected from larvae in a tube containing a few crystals of phenylthiourea at 4°C by cutting an abdominal leg. The hemolymph was immediately centrifuged at 4°C at 10,000 rpm for 10 min to remove hemocytes. Protein concentration in the hemolymph and partially purified protease was determined by the method of Lowry et al. (1951). The hemolymph was diluted with 0.05 M phosphate buffer (pH 7.0) to get a similar protein concentration (per ml) as the partially purified protease. The partially purified protease was mixed with *A. assama* hemolymph in ratios of 1:1, 1:2 and 2:1 and incubated at 30°C for 60 min. Partially purified protease alone and hemolymph alone were incubated under similar conditions as controls. Following incubation, appropriate amounts of SDS-

sample buffer containing 5% mercaptoethanol were added and heated to 100°C for 3 min before application to the gel. The incubated samples were loaded in the well in appropriate volumes maintaining the original ratio of protease and hemolymph. A mixture of standard protein markers was also applied to the gel (10%).

Larval mortality following exposure to the protease was determined. A total of 320 fifth-instar larvae were used for this experiment. Four groups of 40 larvae each (denoted Groups I - IV) were treated with the partially purified protease while four other groups served as respective controls and were treated with 0.05M phosphate buffer (pH 7.0). Individual larvae in Groups I-IV were injected at the muscular base of the third abdominal leg with either 2.5 (Gp. I), 5.0 (Gp. II), 10 (Gp. III) and 25 (Gp. IV) µg partially purified protease using a 10 µl Hamilton syringe. Controls were injected with buffer. Larval mortality was recorded 22 h after treatment. This experiment was replicated three times with the same number of larvae for statistical analysis.

Hemolymph was collected as previously described from protease-injected larvae and from the respective controls at 6 h and 16 h after injection. The hemolymph was stored at –20°C after removal of hemocytes by centrifugation. Protein concentration of these hemolymph samples was estimated (in five replicas for statistical analysis) by the method of Lowry et al. (1951) and subjected to 10% SDS-PAGE to characterize the effect of partially purified protease on protein profile of silkworm hemolymph.

All data were statistically analyzed and statistical analysis of differences between means of injected and control groups was made using Student's 't' test.

Results and Discussion

Pseudomonas aeruginosa is an opportunistic pathogen with a wide host range including plants, insects and other animals (Nicas and Iglewaski 1986). Virulence of *P. aeruginosa* is reportedly multifactorial due to its production of a large number of enzymes and toxins (Liu 1974, Nicas and Iglewaski 1986). Most strains produce several extracellular proteases (Morihara 1964, Kreger and Griffin 1974, Wretlind and Wadstrom 1977). Alkaline protease and elastase have been purified and characterized (Homma 1980, Wretlind and Pavlovski 1983).

The protease that we studied produced a clear zone of hydrolysis when *P. aeruginosa* strain AC-3 was grown in a media containing casein. Maximum protease production in the broth occurred 60 h after incubation (Fig. 1). The specific activity was 10.2 ± 1.57 units per mg protein. Enzymatic activity peaked at 30 min of incubation with the reaction mixture. Thereafter, the activity gradually declined (Fig. 2).

Maximum protease fraction from the 60 h grown *P. aeruginosa* culture broth extract was precipitated at 50% and 70% acetone concentration. As such, in all further experiments the 30% acetone precipitated protein was discarded and the supernatant was brought directly to 70% acetone concentration, the precipitate of which was used for further studies.

The partially purified protein was subjected to 10% SDS-PAGE. The main band observed in the gel (Fig. 3) has been considered to be that of the protease. However, two more faint bands were observed. Such extra bands also were observed by Wretlind and Wadstrom (1977). By plotting the Rf values of the standard protein markers against their respective molecular weights, the molecular weight of the partially purified protease band was calculated to be approximately 35,000 da. Wretlind and Wadstrom (1977) reported that the molecular weight of Protease II was 23,000 ± 5,000. It was one of the three proteases produced by *P. aeruginosa* strain PAKS-I.

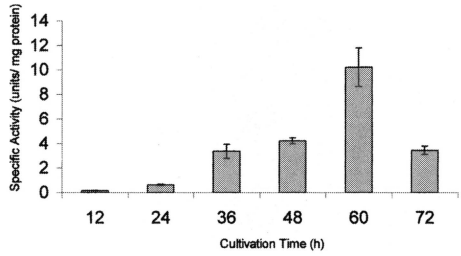

Fig. 1. Amount of protease recovered from media broth supporting growth of *P. aeruginosa* strain AC-3 over time. Values are mean ± S.D. of 3 replications.

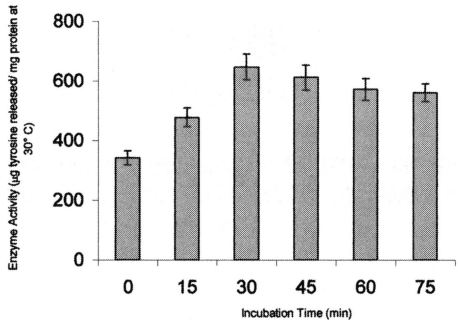

Fig. 2. Incubation time for reaction mixture and protease activity of *P. aeruginosa* strain AC-3 cultivated for 60 h in broth media. Values are mean ± S.D. of 3 replications.

Morihara et al. (1965) estimated the molecular weight of fraction II (corresponding to Protease II) to be 39,500 da. The reason for this discrepancy is not known, but differences in the strains of *P. aeruginosa* studied, the methods for purifying the enzymes and the method for determining molecular weight are probable causes.

The protein profile of partially purified protease incubated *in vitro* with larval hemolymph at different ratios revealed several differences between the control hemolymph as compared with the protease-treated hemolymph (Fig. 4). Protein 1 and the bands between this protein and protein 2 were found to be totally absent in the incubated samples. Intensity of protein 2-7, 10-12 decreased with increased concentration of protease. In hemolymph treated with 2X protease, the proteins 2 and 6 were not observed, and bands 8 and 9 also were not detected in the treated hemolymph. A band corresponding to that of the partially purified protease also was observed in the treated hemolymph and probably represents the protease present in the treated hemolymph. The low intensity or the complete absence of some of the protein bands in the treated samples suggests that the protease present in them partially or fully degraded the respective original proteins (e.g., the band below protein 9). Some extra bands that had been observed in the protease-treated hemolymph samples suggest

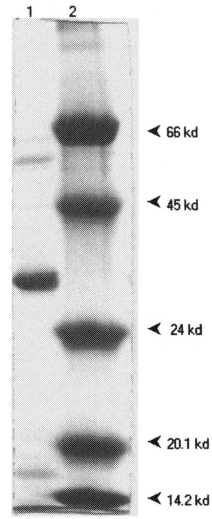

Fig. 3. SDS-PAGE protein profile of the partially purified protease released by *P. aeruginosa* strain AC-3 (*Lane 1*) and standard protein markers for comparison (*Lane 2*).

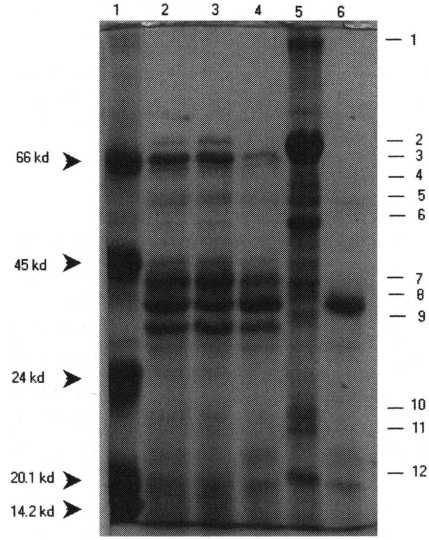

Fig. 4. SDS-PAGE protein profile of incubated mixture of *A. assama* hemolymph and partially purified protease of *P. aeruginosa* strain AC-3. *Lane 1:* Standard molecular markers, *Lane 2, 3 & 4:* 1:1, 1:2 & 2:1 partially purified protease and hemolymph mixtures, *Lane 5:* Hemolymph without protease treatment, *Lane 6:* Partially purified protease.

these are breakdown products of the original bands observed in untreated hemolymph.

Muga silkworm fifth instars injected with either 10 or 25 µg of the partially purified protease died within 16 h of injection (Table 1). At 22 h after injection, 20% and 55% of larvae injected with 2.5 and 5.0 µg of protein, respectively, were dead. The difference in mortality level between the injected and control groups was found to be statistically significant. The surviving larvae within these two groups of protease injected-larvae produced flimsy and malformed cocoons. The reason for the detrimental effect of the protease on the silkworm larvae is not exactly known but may be due to the inhibitory action of protease on biologically important proteins/enzymes of the silkworms. This is further supported by our observation of an indirect statistically significant relationship of hemolymph protein concentration with increasing amounts of protease injected (Table 2). The mortality level of the controls was 0. Death of those larvae injected with 10 and 25 µg of protease further supports the detrimental effect of the applied protease on the silkworm metabolism.

As already mentioned *A. assama* larvae were treated with four concentrations of protein (partially purified protease) and their respective controls were treated with 0.05M phosphate buffer (pH 7.0). Fig. 5 shows the hemolymph protein profile of the larvae of groups I-IV and their respective controls at 6 h after treatment and that of groups-I and II and their controls at 16 h after treatment. Larvae belonging to groups-III and IV were dead at this stage. The bands obtained with SDS-PAGE separation of treated hemolymph have been denoted as "protein". Only the prominent and visible bands observed in the gel photograph have been marked (1-5). The intensity of protein 1 appeared to increase with increasing concentration of applied protein (i.e., from 2.5 to 5 µg). However, in the hemolymph of the larvae treated with 10 and 25 µg, the protein could not be detected suggesting its degradation. At 16 h after injection the intensity of the protein band was low in the 5 µg protease-treated larvae indicating its partial degradation. The intensity of the protein 2 gradually decreased from larvae injected with 2.5 and 5.0 µg at 6 h after injection. At 16 h a further decrease in intensity was observed in these treatments. Several new protein bands in the region of protein 2 were observed in all the injected larvae; these were most prominent in the 5 µg protease-treated larvae. The appearance of the extra bands suggests that these might be the degradation products of the biologically-active proteins. The protein 2 may be a component of heavy density lipoprotein (Hughes et al. 1983) or larval

Table 1. Mortality of *A. assama* fifth instar larvae after injection with specified concentration of partially purified protease released by *P. aeruginosa* strain AC-3

Time (h)	Protease concentration				
	0 µg	2.5 µg	5 µg	10 µg	25 µg
0	0	0	0	0	0
6	0	0	0	20 ± 1.16*	32 ± 1.00*
16	0	0	0	40 ± 0.00*	40 ± 0.00*
22	0	8 ± 0.58*	22 ± 1.53*	40 ± 0.00*	40 ± 0.00*

Each value is mean ± S.E.M. of three replications. *$P < 0.05$.

Table 2. Protein concentration (mg/ml) in hemolymph of *A. assama* fifth instar larvae at different time interval after injection of specified concentration of partially purified protease released by *P. aeruginosa* strain AC-3

Time (h)	Control	Treated (2.5 µg)	Control	Treated (5 µg)	Control	Treated (10 µg)	Control	Treated (25 µg)
	Group-I		Group-II		Group-III		Group-IV	
0	14.66 ± 0.65	14.92 ± 0.29	15.06 ± 0.21	14.99 ± 0.78	14.91 ± 0.96	14.99 ± 0.76	15.00 ± 0.23	15.25 ± 0.34
6	15.05 ± 0.25	14.95 ± 0.26	15.18 ± 0.23	14.65 ± 0.57	14.89 ± 0.10	12.52 ± 0.66*	15.03 ± 0.23	10.82 ± 0.73*
16	15.19 ± 0.19	14.54 ± 0.31*	15.22 ± 0.35	14.12 ± 0.31*	—	—	—	—

Each value is mean ± S.D. of five replicatins. *$P < 0.05$.

storage proteins. Protein 3 could not be detected in the hemolymph of any of the treated larvae at either 6 h or 16 h. The intensity of protein 4 also decreased with increasing concentration of protease such that it was not detected in the 10 and 25 µg treated hemolymph. The appearance of a number of new protein bands in the treated larvae in the low molecular weight region further supports the hypothesis that the protease partially degrades biologically-important proteins of *A. assama* larvae. Protein 5 was observed to be present in all the control and treated sets.

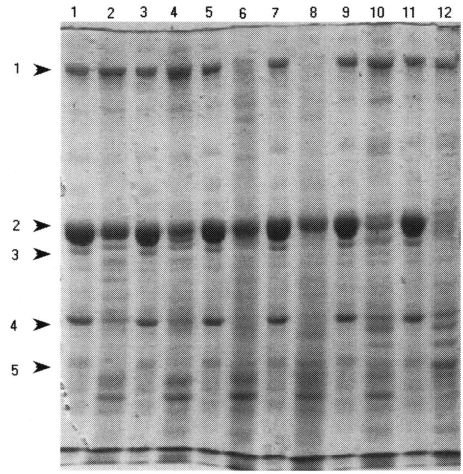

Fig. 5. SDS-PAGE protein profile of hemolymph of control and partially purified protease injected. *A. assama* larvae. *Lane 2, 4, 6, & 8:* 6 h after injection with 2.5, 5.0, 10 and 25 µg partially purified protease, respectively. *Lane 10 & 12:* 16 h after injection with 2.5 and 5.0 µg partially purified protease, respectively. *Lane 1, 3, 5, 7, 9 & 11:* Respective control larvae.

Pseudomonas aeruginosa protease reportedly degrades a broad spectrum of biologically important proteins in humans (Doring et al. 1981). The proteases can cause hemorrhages in tissues and internal organs. Purified proteases are capable of causing necrosis and ulcerating lesions when injected sub-cutaneously, pulmonary lesions when injected by intravenous, intratracheal or intranasal routes, and hemorrhage and necrosis in the gastrointestinal tract when injected extra peritoneally. It seems likely that the lethal activity of *P. aeruginosa* proteases is due to the hemorrhages they illicit in internal organs (Liu 1966).

It is evident from our study that the protease, partially purified from *P. aeruginosa* strain AC-3 is lethal to *A. assama* larvae. It is further apparent that the protease partially or fully degrades biologically important proteins of the silkworm larvae. Additional research is necessary to ascertain the actual lethality of protease on silkworm larvae and its role in the flacherie disease of muga silkworm.

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