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Screening the efficacy of multiple buffers on the optimization of *in vitro* activity of prophenoloxidase (PPO) enzyme in both healthy and pebrine infected muga silkworm larvae

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Abstract:
Hemolymph of insects possesses the properties that decipher the capabilities of immune responses and other vital characteristics to survive against diseases. Humoral responses generally comprise the prophenol oxidase activating system (PPO-AS) while cellular responses include the involvement of hemocytes causing coagulation, phagocytosis, nodule formation and encapsulation (Gillespie *et al*., 1997). In an attempt to isolate and characterize PPO in muga silkworm, hemolymph of 5th instar larvae of the insect (both healthy and pebrine infected) were collected through established methodology (Goudru et al., 2013). The efficacy of potassium phosphate, Tris-Cl, sodium phosphate and sodium cacodylate buffers were tested in presence of anticoagulant solution containing 30mM sodium citrate in various proportions to determine the maximum PPO activity of the extracts in presence of L-DOPA as substrate. Sodium cacodylate buffer restored the maximum PPO activity by inhibiting melanization of the hemolymph thereby converting L-DOPA to dopaminechrome; optimum between concentrations of 5mM to 6mM L-DOPA. We propose that sodium cacodylate buffer supplemented with anticoagulant solution containing 30mM sodium citrate is best suited for determining PO activity and can be used for long-term storage of *Antheraea assamensis* Helpher hemolymph *in-vitro*.

Key words: Prophenoloxidase, *Antherarea assamensis*, sodium cacodylate buffer, anticoagulant solution.

Introduction:

Hemolymph of insects possesses the properties that decipher the capabilities of immune responses and other vital characteristics to survive against diseases. It is established that insect hemolymph triggers the sequential responses of an innate immune system which involve pattern recognition, proteolytic cascades, signal transduction, cellular reactions and induction of antimicrobial peptides (reviewed by Gillespie et al., 1997; Engstrom, 1999; Hoffmann et al., 1995). The activation of

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proteolytic cascades leads to melanization via a prophenoloxidse enzyme reaction resulting in hemolymph coagulation. This results in a major hurdle to process and store insect hemolymph *in-vitro* for physiological, biochemical and molecular studies.

Phenoloxidase (PO) plays an important role in the defense response to the foreign invaders, e.g. pathogens and parasites, in the formation of melanin as well as in cuticle sclerotization in insects (Ashida *et al*, 1990). PO is synthesized as a zymogen called prophenoloxidase (PPO), which can be activated by specific proteolysis. When insects are infected by microorganism, PPO activation is elicited by microbial cell surface components, such as, lipopolysaccharide (LPS), peptidoglycans, β-1,3-glucose (Lee *et al*, 2000). PO causes massive deposition of melanin at the wound / infected site thereby preventing loss of hemolymph and blocking the entry of opportunistic invading pathogens. Active Phenol Oxidase (PO) carries out hydroxylation of monophenols to produce diphenols and oxidizes them to quinones which can be studied *in-vitro* (Cerenius and Soderhall, 2004). The inactive form of PO is Prophenol Oxidase (PPO) which circulates in insect hemolymph and can be activated by certain agents to PO through proteolytic cleavage. The role of activators and inhibitors in PPO activation are crucial in PO cascade and are of great immunological importance (Serenius and Soderhall, 2004).

The Indian golden silkmoth (*Antheraea assamensis* Helpher), popularly known as muga silkmoth, is endemic to northeastern part of India (Arunkumar *et al*., 2009) and is famous for the golden silk that has enormous economic value. However the population of the insect is fast declining in recent times due to habitat destruction and natural diseases like pebrine caused by the microsporidium protozoan *Nosema assamensis* (Hatakeyama and Hayasaka, 2003). It is assumed that the population of the species can be restored via activation of the immune system which is germ-line encoded and is classically divided into innate and adaptive immunity. The innate immune system consists of effector events viz. humoral and cellular (Schmid-Hempel, 2005). Humoral responses generally comprise the prophenol oxidase activating system (PPO-AS) while cellular responses include the involvement of hemocytes causing coagulation, phagocytosis, nodule formation and encapsulation (Gillespie *et al*., 1997).

The role of cellular immune responses including activation of the PPO cascade in muga silkworm in response to microsporidian *Nosema assamensis* infection needs to be studied. An effective approach to enhance PO activity in *A assamensis* shall provide a possible clue to overcome infectious diseases like pebrine caused by microsporidian *Nosema assamensis* which reportedly destroy large populations of the silkmoth. Notably, no proper study has been documented on preparation and storage of *A assamensis* hemolymph without effecting its properties, which has stood as the key obstacle to perform molecular experiments on the silkmoth. With these aims we have chosen this problem to find out a suitable buffer system to restore *A assamensis* PO activity in-vitro.

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Materials and methods:

Collection of hemolymph from A assamensis larvae

A total of twenty 5th instar larvae (10 normal and heallthy while 10 were pebrine infected) of *A assamensis* were collected, carried to laboratory, washed and disinfected with 0.1% H_2O_2 and hemolymph was collected into four fractions by excising the forelegs of the larvae. A total of 8 fractions were recovered for the studies.

Preparation of anticoagulant solution

Anticoagulant solution was prepared following the methodology of Kwon *et al* (1997) with modifications by adding 30mM trisodium citrate. pH was adjusted to 8.0 with 1M NaOH and the solution was filter sterilized with Whatman 0.45mm syringe filter before use.

Storage of hemolymph fractions in tested buffers

In order to test the efficacy of different buffer fractions for storing the collected hemolymph, four different buffer preparations were done following previously established protocols (Feng & Fu, 2004). These included 0.2M cacodylate buffer, 50mM sodium phosphate buffer (pH 6.0), 80mM pottasium phosphate buffer (pH 6.5) & 0.2M Tris-Cl buffer (pH 7.2) (Shaiemma *et al*., 2012).

The hemolymph collected was stored in the prepared buffers as fractions viz **fraction A** which comprises of 500µl of Cacodylate buffer, 500µl anticoagulant solution and 500µl hemolymph; **fraction B** which comprises 500µl of 80mM Pott. Phosphate buffer (pH 6.5), 500µl anticoagulant solution and 500µl hemolymph; **fraction C** which comprises of 500µl of 50mM Sodium phosphate buffer (pH 6.0), 500µl anticoagulant solution and500µl hemolymph; **fraction D** which comprises of 500µl of 0.2M Tris-Cl buffer (pH 7.2), 500µl anticoagulant solution and 500µl hemolymph

The diluted hemolymph collected as above were centrifuged at 8000 rpm for 30 minutes in a refrigerated centrifuge, and the supernatant was separated from the cell pellet. This Hemolymph Supernatant (HS) was stored at -20^{0}C containing 0.2M Sodium azide (NaN_3) and was later total protein content of all the four fractions were measured by Folin Lowry method using BSA as standard curve (Lowry et al., 1951)

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Assay and determination of PO activity of separated hemolymph

Samples (HS Fractions A to D) were pre-incubated at 25^{0}C for 30 minutes for determining PO/PPO activity. 900µl of 50mM sodium phosphate buffer was added to 60µl of the samples, 5ml of 10% Cetyl Pyridium Chloride (CPC) and 60µl of 2mM L-DOPA solution. The reaction mixture was incubated further for 10 minutes and increase in absorbance due to oxidation of dopamine was monitored through scanning OD from 450-500nm in a UV-VIS spectrophotometer (Eppendorf) as was mentioned by Feng & Fu (2004). One unit of PO activity was calculated as increment in the rate of absorbane and an increase of 0.001/min at the most stable wavelenght (Laughton & Siva-Jothy, 2010).

Optimization of maximum PO activity in presence of varying concentrations of L-DOPA

For the studies, the HS fractions of Normal and Diseased Fractions-A were subjected to 50-70% ammonium sulfate precipitation followed by dialysis in 20mM Tris-Cl buffer (pH 7.0) for 12 hours at 4^{0}C. 500 µl of 33.3mM APMSF was added to all the fractions of HS before dialysis as per methodology of Asano and Asida (2001). Besides the HS fractions were centrifuged at 20000 rcf (13722 rpm) for 30 minutes at 4^{0}C before loading the contents onto dialyzing membrane (Genetix, 70-100 kD cut). The dialyzed fractions were later subjected for determination of maximum PO activity under varying concentrations of L-DOPA ranging from 2mM to 10mM following the methodology as mentioned above.

Results:

Estimation of total protein content:

Total protein content of the collected hemolymph was measured by Folin Lowry method. The absorption maxima were measured at 660 nm in a UV-VIS septrophotometer (Eppendorf). OD values were converted to protein content (µg/ml) from BSA standard curve prepared at concentration 10 µg/ml.

Protein content is found to be highest in fraction A in both normal and diseased larvae followed by fraction D.

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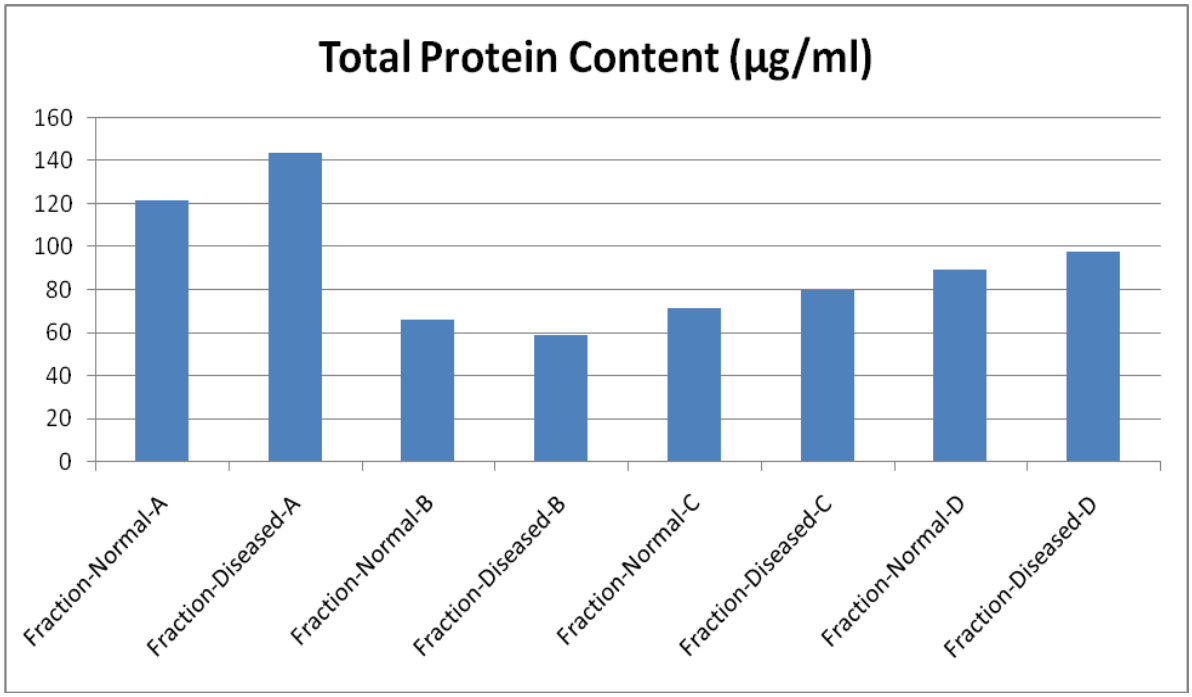

Fig1: Total protein content of hemolymph of normal and diseased larva stored in four different buffers

Assay of Phenoloxidase activity:

Among the four fractions phenoloxidase activity is also highest in fraction A in both normal and diseased larvae, similar to the concentration of the protein.

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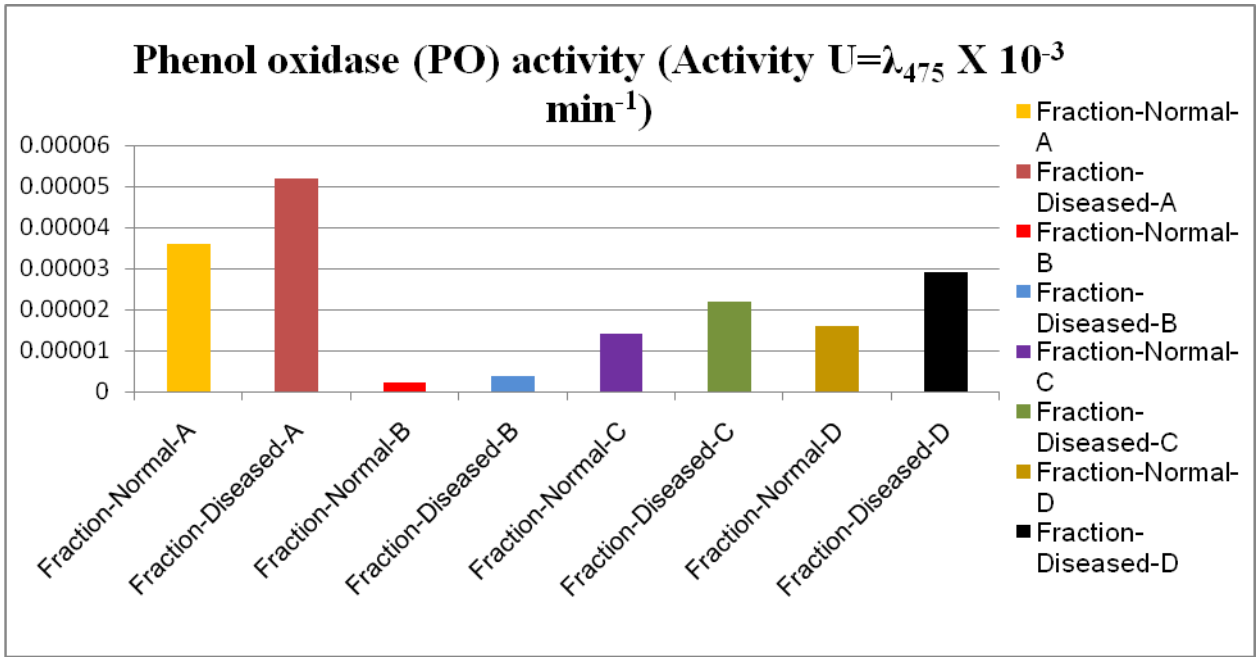

Fig 2: PO activity of hemolymph of normal and diseased larva stored in four different buffers.

PO activity of Diseaded and Normal larvae hemolymph fractions (Fraction A) under varying concentration of L- DOPA

The Hemolymph Supernatant (HS) fractions from normal and diseased larvae were subjected to NH_4SO_4 precipitation followed by dialysis in Tris-Cl buffer prior to the PO activity test .PO activity at increasing concentrations of L-DOPA were tested only on Fraction-A hemolymph of both normal and diseased larvae. The other Fractions were not tested as they did not show appreciable PO activity in the previous study. Between 5-6 mM L-DOPA concentration (which acted as substrate for the enzyme) it showed maximum PO acivity.

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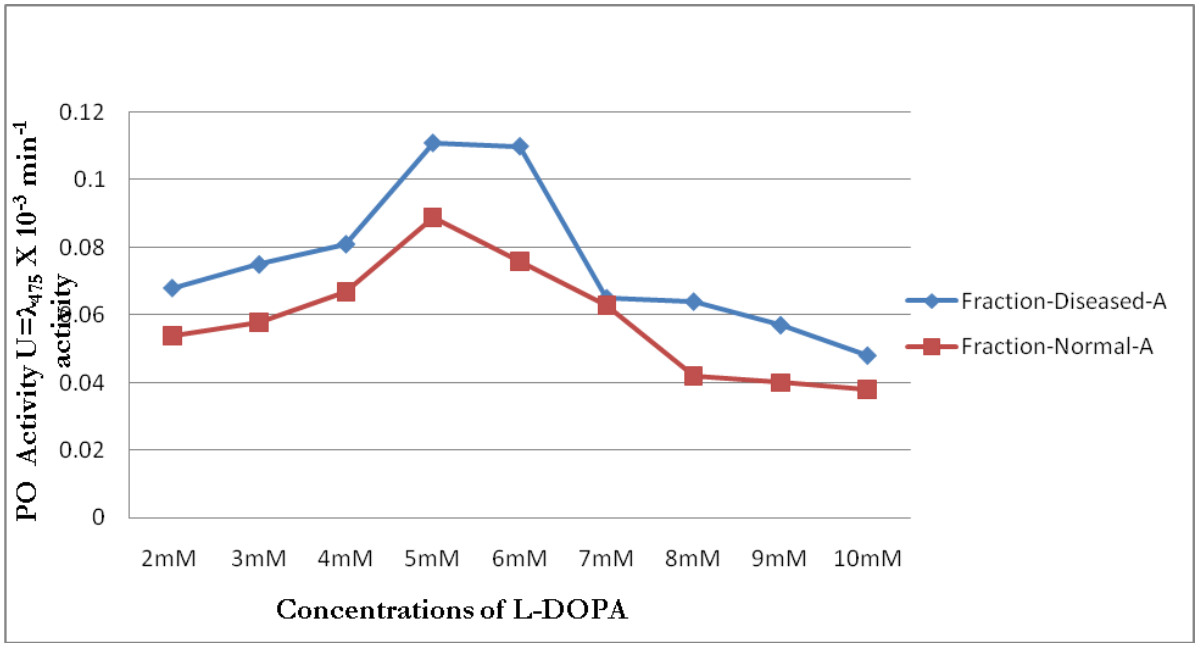

Fig 3: PO activity of Diseaded and Normal larvae hemolymph fractions (Fraction A) under varying concentration of L- DOPA

Discussion and Conclusion:

Rearing and cultivation of *A assamensis* has been a part of traditional practice of the region and for many native inhabitants; its cultivation contributes to economic stability, sustainable livelihood options and income generation. *A assamensis* has been widely exploited for commercial benefit and its wild population has tremendously declined in recent past due to deforestation, rapid habitat fragmentation, depletion of host plant cover, climate change and anthropogenic pressure, whilst various tropical diseases caused by fungal, bacterial, viral and protozoan infestations have dwindled populations both in the wild and the cultivated forms. The silkworm remains scientifically unexplored compared to the other silk producing insects including *Bombyx mori*.

Our experiments revealed that Cadodylate buffer is best suited for restoration of *in-vitro* PO activity in collected hemolymph from both normal and pebrine infected larvae of *A assamensis*. We could

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Index in Cosmos
May 2018 Volume 8 Number 5
UGC APPROVED

overcome the process of rapid melanization of larval hemolymph by addition of anticoagulant solution to the buffer which was reported in other arthropods but was not reported in silk moths including *Bombyx mori*. We also noticed that addition of trisodium citrate at conc. 30mM to the anticoagulant solution efficiently inhibits melanization upto ~90% and restores PO activity *in-vitro*. The total protein content of hemolymph fractions stored in cacodylate buffer appeared to be maximum in both normal and diseased individuals. Our experiments confirmed that maximum activity of PO of cacodylate stored hemolymph fractions (Fraction-A) from both normal and diseased larvae was optimum between 5mM to 6mM L-DOPA concentrations. Maximum PO activity of all the tests performed was recorded at 475nm whilst the optimum temperature for maximum enzyme activity was found to be 30^0C.

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Index in Cosmos
May 2018 Volume 8 Number 5
UGC APPROVED

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Index in Cosmos
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