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RESEARCH ARTICLE

PHENOLOXIDASE ACTIVITY IN HAEMOLYMPH OF SILKWORM *BOMBYX MORI* L. DURING THE DEVELOPMENT OF FUNGAL PATHOGEN *BEAUVERIA BASSIANA* (BALS.) VUILL.

Rajitha. K and Savithri. G

Department of Sericulture, Sri Padmavati Mahila Visvavidyalayam, Tirupati-517502, Andhra Pradesh, INDIA

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ABSTRACT

Day to day changes in the activity of phenoloxidase was examined in the haemolymph of 5th instar silkworm *Bombyx mori* L during the progress of fungal pathogen *Beauveria bassiana*. Increased trend of phenoloxidase activity was recorded in haemolymph of both inoculated (0.491 to 0.5 µ moles/mg protein/min) and control (0.456 to 0.473 µ moles/mg protein/min) from the 1st to 3rd day of the instar, then the enzyme activity was declined in the rest of the 5th instar in treated (0.499 µ moles/mg protein/min to 0.142 µ moles/mg protein/min) and control (0.413 µ moles/mg protein/min to 0.200 µ moles/mg protein/min). Significant elevation of phenoloxidase (PO) activity was recorded up to 4th day of the instar then the drastic reduction of the enzyme activity was observed in *Beauveria bassiana* inoculated silkworm larvae compared to control.

INTRODUCTION

Diseases in silkworm *Bombyx mori* are fairly common in occurrence and are serious in inflicting losses. Among the many constraints that influence the success of cocoon production, the menace of disease is the prime one. The major diseases affecting mulberry silkworm *Bombyx mori* can be grouped under four major categories, namely the microsporidian disease, viral diseases, bacterial diseases and fungal diseases. Among the diseases of silkworm, white muscardine caused by *Beauveria bassiana* inflicts heavy economic loss to the sericulturists in India. Muscardine is one of the contagious diseases, which is causing loss to 5-50 percent in total loss due to diseases.

Silkworm *Bombyx mori* is very much susceptible to diseases due to centuries of domestication. The remarkable evolutionary success of insects is partly due to their ability to build up a sophisticated, effective, and highly adaptable defense system against numerous microorganisms, including pathogenic fungi. The host defense system of insects relies on several innate cellular and humoral reactions that are tightly interconnected. Upon the invader's breaching of the physical barriers, the immediate onset of enzymatic cascades leads to localized blood clotting and melanization, involving the production of cytotoxic molecules. In this response, a pivotal role is played by the prophenoloxidase (proPO)- activating system. Phenoloxidase (PO) is a vital enzyme involved in a number of crucial processes, such as defense, wound healing, sclerotization, and pigmentation. Since active PO generates deleterious quinonoid compounds, most insects preserve this enzyme in the inactive form and activate it upon necessity. The PO detected in both cuticle and haemolymph is derived from a proenzyme, prophenoloxidase. The PO cascade takes part in the melanization of haemocytes attached to the surface of the parasite (Chain and Anderson, 1982; Rizki and Rizki, 1984; Takahashi and Enomoto, 1987; Pech and Strand, 2000).Phenoloxidose is available in the form of non-active proenzyme prophenoloxidase, in the cuticle and haemolymph of insects (Johansson and So¨derha¨ ll, 1995; Ashida and Brey, 1997; Sugumaran, 2002) and PO activity in the haemocytes, especially in granulocytes and oenocytoides, was observed (Kopac˘ek *et al .*, , 1995; Ashida and Brey, 1997).

MATERIALS AND METHODS

For the investigation silkworm hybrid of PM x CSR_2 was selected. Silkworm larvae were brushed and reared under laboratory conditions according to Dandin *et al.,* (2003). Immediately after fourth moult i.e. on the first day of the fifth instar, the larvae were inoculated, by dipping in fungal spore suspension (2.15 x 10^6 spores/ ml @ 50 ml/100 worms for 45 sec). The larvae treated with double distilled water were used as control. After 24 hours of the induction of fungal pathogen haemolymph was collected everyday i.e. 2nd day to 7th day of the 5th instar silkworm larvae into pre-chilled centrifuge tubes with a pinch of thiourea by clipping third pair of abdominal legs of silkworm larvae for day to day analysis. Phenoloxidase activity was examined by following the method of Mason, (1947) using a spectrophotometer. Recorded data of the study was statistical analysied by using t-test.

RESULTS AND DISCUSSION

The results (Table-1 and Graph-1) showed the increased trend of phenoloxidase activity in both inoculated (0.491 to 0.5 µ moles/mg protein/min) and control (0.456 to 0.473 µ moles/mg protein/min) from the 1st to 3rd day of the instar, then the enzyme activity was declined in the rest of the 5th instar in treated (0.499 µ moles/mg protein/min to 0.142 µ moles/mg protein/min) and control (0.413 µ moles/mg protein/min to 0.200 µ moles/mg protein/min). Compared to control the enzyme activity was significantly enhanced up to 4th day of the infection, then the enzyme activity was declined significantly in the rest of the instar.

* Corresponding author: Rajitha. K
Department of Sericulture ,Sri Padmavati Mahila Visvavidyalayam ,Tirupati-517502, Andhra Pradesh, INDIA

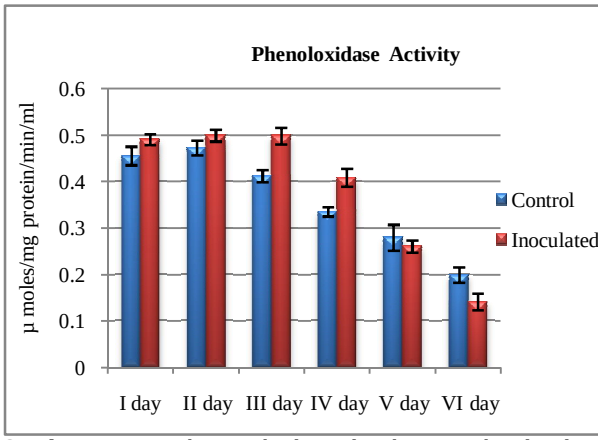
Table 1 Day to day changes in phenoloxidase activity (µ moles/mg protein/min/ml) in the haemolymph of silkworm *Bombyx mori* L. inoculated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill. with reference to control during 5th instar.

Treatments	Days of 5th instar					
	I	II	III	IV	V	VI
Control	0.456± 0.020	0.473± 0.016	0.413± 0.013	0.336± 0.010	0.280± 0.028	0.200± 0.017
Inoculated	0.491± 0.012 ****	0.5±0. 013 ****	0.499± 0.018 ****	0.409± 0.019 ****	0.261± 0.013 ****	0.142±0.0 18 ****

Mean±Standard Deviation; NS = Not Significant; *P≤0.05, **P≤0.02, ***P≤0.01, ****P≤0.001

Initial enhancement of enzyme activity may be due to the consequence of the invasion of fungal pathogen into the host haemolymph. In lepidopterans, proPO mostly appears in haemolymph. In other groups such as *locusts* and cockroaches, proPO stored in haemocytes until a pathogen induces its release (Brehe´lin *et al.*, 1989; Durrant *et al.*, 1993). PO-generated quinones may serve as toxic metabolites that might be harmful to the intruders (Ashida and Yamazaki, 1990; Kopacek and Sugumaran, 1998). Products of PO activity, i.e. melanin and its oxidized precursors have been shown to have fungistatic activity (St. Leger *et al.*, 1988). This enzyme is responsible for the activation of melanogenesis in invertebrates (Sussman, 1949; Wyatt, 1961). Activation of PO results in the production of 'sticky' proteins which have been implicated in the recognition of non-self (Söderhäll *et al.*, 1979). Several studies have shown that PO levels are elevated in response to natural fungal infection or injection of fungal components (Gillespie *et al.*, 2000a). Invaders that are able to penetrate successfully into the insect hemocoel will face a battery of cell defenses, including the phagocytosis of small pathogens and the formation of multicellular layers that encapsulate large intruders by the blood cells. Encapsulation and melanization effectively limit the damages caused by invading organisms by forming a physical barrier between the self and non-self (Sugumaran, 1998).

Decreased enzyme activity in the later stage of infection might be due to suppression of host enzyme activity by releasing inhibitor factors by the invading fungal pathogen. Parasitoids are able to suppress the phenoloxidase system through the inhibition of protease activity, blocking of pattern-recognizing proteins and destruction of immunocompetent cells (Vinson, 1990; Brehelin, 1990; Shelby *et al.*, 2000). It may also be due to decreased intake of food due to disease. The cost of production and maintenance of the PO system (including the proPO-activating system) is likely to be high for two reasons. First, the main compound of the proPO-activating system–tyrosine– is obtained from phenylalanine, which can only be obtained from ingested food (Chapman, 1998). Second, melanin, a final product of proPO-activating system, is a nitrogen-rich compound, which may require substantial nitrogen or protein investment for its synthesis (Blois, 1978; Lee *et al.*, 2008). Thus, production and maintenance of the proPO-activating system is dietary-dependent. Several research studies have also found that a protein-based diet is needed for an optimal proPO-activating system. In support of this investigation Srygley et al. (2009) reported that in the Mormon cricket *Anabrus simplex* Haldeman, animals fed a high-protein diet showed higher values of total PO activity than animals fed a high-carbohydrate diet. Related results were found in the caterpillars *Spodoptera exemptra* Walker and *Spodoptera littoralis* Boisduval: infected animals fed a higher protein: carbohydrate ratio had more PO activity and survived for longer than those fed a lower protein:carbohydrate ratio (Lee *et al.*, 2006; Povey *et al.*, 2009). A possible explanation for these results is that protein content directly affects the production of amino acids, which can be used for the synthesis of several compounds of the proPO-activating system, including phenoloxidase (Abisgold and Simpson, 1987).



Graph-1 Histogram showing the day to day changes inphenoloxidase activity (µ moles/mg protein/min/ml) in the haemolymph of silkworm *Bombyx mori* L. inoculated with fungal pathogen *Beauveria bassiana* (Bals.) Vuill. with reference to control during 5thinstar

Depression of immune reactions is one of the main mechanisms governing the outcome of relations between a host and an invader. It is therefore reasonable to assume that inhibition of the proPO system, allowing avoidance of host humoral encapsulation, could be a key to successful parasitization. The decline may be due to the immunosuppressive effect of fungal proteins or toxic metabolites. Indeed, destruxins prevent PO production by *locust* haemocytes (Huxham *et al.*, 1989), probably by destroying the cells that produce proPO (Cerenius *et al.*, 1990). Metallothionein, a fungal protein selectively binding heavy metal ions isolated from *Aspergillus niger*, was found to be a potent PO inhibitor (Goetghebeur and Kermasha, 1996). Two proteins present in *Galleria mellonella* haemolymph, i.e. ApoLp-III, which improved cecropin activity, and Gm protein-24, both playing a role in the activation of the proPO cascade (Park *et al.*, 2005), may be the targets for fungal immunosuppressive effectors.

Bogus et al. (2007) reported that exposition to *Conidiobolus coronatus* slightly induces PO activity of *Conidiobolus vicina.* In contrast, *Conidiobolus coronatus* infection has no influence on the PO activity of *Diprion pini*, while in *Galleria mellonella the* PO activity dramatically drops. Depressed rates of *Galleria mellonella* haemolymph PO activities were also found in larvae parasitized by the entomopathogenic nematode *Steinernema feltiae (*Brivio *et al.*, 2002) or infected by *Metarhizium anisopliae* (Slepneva *et al.*, 2003). Similarly, parasitism of *Manduca sexta* larvae by the braconid wasp (Beckage *et al.*, 1990) as well as *Metarhizium anisopliae* infection of *Schistocerca gregaria* (Gillespie *et al.*, 2000b) depresses the rates of haemolymph PO activity.

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