



INDUCED PHENOLOXIDASE PROFILES IN SILKWORM *Bombyx mori* (L.) UNDER BIOTIC STRESS

Vishnu Priya S^{*1}, Somasundaram P²

^{*1}Assistant Professor, Department of Biotechnology, Mahendra Arts and Science College, Namakkal, India

²Central Sericultural Germplasm Resource Centre, Central Silk Board, Hosur, India



Abstract:

*Phenol oxidase (PO) is one of the stress enzyme protein in living organism. The conversion of Pro-PO into an activation form of PO required a stress protein. In the present study has emerged with the novel finding of induced phenoloxidase was identified under bacterial endotoxin viz., Lipopolysaccharide (LPS) activity using silkworm *Bombyx mori* as an animal model. The PO enzyme plays an important role for insect survival during pathophysiological conditions. The enzyme activity was analyzed from ten different silkworm races with two phenolic substrates viz., L-Dopa and Dopamine by Native-PAGE. The bacterially induced PO was found in hemolymph and midgut of silkworm, PO3 were induced by LPS injection. In control PO1 & PO2 are non-bacterially induced protein having the molecular weight of 72 and 71. The results shown that there is no substrate specificity and similar functional activity was found in hemolymph and midgut under pathogenic condition. It was observed that bacterially inducible PO clearly differed from non-inducible PO (control). At final observation of induced isozymes of PO in the haemolymph and midgut system of tolerant silkworm races points out the existence of biochemical immunity against biotic stress of LPS. This is the first report to document the silkworm immunity under the LPS toxin in different silkworm races to identify the tolerant and susceptibility against a biotic stress.*

Keywords: Bacterial Endotoxin; *Bombyx Mori*; Phenol Oxidas; Immunit; Stress Induced Isozyme.

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1. Introduction

For evaluation of cellular and humoral parameters of the immune response of silkworm *Bombyx mori*, the development of simplified procedures has played a vital role for the development of immunoassays. The quantification of different cellular and humoral parameters of the immune response of silkworm will give a clue for silkworm health status. Insects produces a immune proteins when stimulated by pathological infections stress proteins [1,2,3]. Phenoloxidase (PO) activities have been considered as potential markers [4, 5, 6, 7, 8]. PO and

Antibacterial immune proteins have been well characterized and it can be considered as an environmental marker [6].

It was reported that phenoloxidase involve in host defence mechanism to detoxify the toxic substances produced by microbial infections [9]. Takahiro and Kato [4] reported the induced carboxyl esterase activity in one silkworm using *E.coli* toxin. Phenoloxidase produces indole groups during biotic stress and subsequently polymerized to melanin. The enzymatic reactions in turn produce a set of intermediate products such as quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates, which are important during defense against bacterial gram positive and gram negative, fungal, and viral infections. Invertebrate PO requires a complex system of activation and inhibition. Activation and inhibition involve different cell types, PO zymogens, proPO inhibitor enzymes (serpins), signaling molecules (peptidoglycan, membrane lipids, and viral protein segments), and even PO itself. In this study, Phenoloxidase (PO) activity in ten silkworm races were compared before and after Lipopolysaccharide (LPS) injection. It is believed that, this is the first documentary report for finding silkworm immunity in different races under a bacterial endotoxin stress.

2. Materials and Methods

2.1. Test Material

The list of ten selected multivoltine (MV) silkworm races were taken for analyzing bacterial toxicity viz., LPS (Lipopolysaccharide) administration were shown in Table 1.

Table 1: List of ten Selected silkworm races of *Bombyx mori*

S.No.	Race/AccessionNo.	Race /Accession Name
1	BMI 0001	Pure Mysore
2	BMI 0017	Nistari
3	BMI 0009	Kollegal Jawan
4	BMI 0014	OS 616
5	BMI 0056	MY1(SL)
6	BMI 0036	PMX
7	BMI 0034	AP12
8	BMI 0006	Hosa Mysore
9	BMI 0035	A13
10	BMI 0004	TamilNadu White

2.2. Administration of LPS

The ten MV silkworm races are maintained at Central Sericultural Germplasm Resources Centre, Hosur were selected for this study. The 5th instar 4th day larvae were treated with an LPS (*E.coli* 0111:B1) by intravenous injection of saline containing 100g of LPS. In control larvae saline only administrated [4,10,11].

2.3. Sample Collection

Haemolymph and midgut was collected separately for haemolymph and followed by phosphate buffer saline were added (PBS) (PH 7.4). The midgut is grounded and dissolved in extraction buffer (PH 7.2) which was used for further study. The haemolymph and midgut were centrifuged and the supernatant was collected in a separate pre-cooled microfuge tube and stored at -20°C. The protein content was measured according to Lowry et al.[12] Method.

2.4. Biochemical Analysis Phenoloxidase (PO) Isozyme

The samples of haemolymph and midgut of individual ten larvae with and without LPS treated were subjected to electrophoresis under non-denatured conditions (Native-PAGE) on 10% poly acrylamide gel. Electrophoresis was carried out with reservoir buffer (pH 8.3). Then gels were stained with L-DOPA and Dopamine substrate separately [13]. The relative mobility (Rf values) was calculated by their Rf value. The resulted phenoloxidase bands were designated as PO1, PO2, PO3 [8,14,15].

3. Results and Discussions

3.1. Biotic Stress (LPS) Induced Polymorphic Patterns

Phenoloxidase (PO) in the haemolymph and midgut of ten silkworm races of individual larvae with or without LPS injection were analyzed on native PAGE by using phenolic substrates viz., L-Dopa and Dopamine substrate. The haemolymph of LPS-treated PO banding pattern in selected silkworm races showed polymorphism among the selected races. There was 3 different types of PO bands resolved and designated as PO-1, PO-2 and PO-3 based on their diphenolic substrates and Rf value. The haemolymph from larvae injected with LPS had additional one additional PO isoforms PO-3. By using L-Dopa substrate the LPS induced isoforms DAPO3 was observed in Pure Mysore, Nistari, OS-616 and MY1(SL) in LPS treated races and the molecular weight is 46 KDa and non-induced molecular weight viz., 72 and 72KDa respectively (Fig not shown). The induced isoforms of POs viz., DEPO3 invariably present in Pure Mysore, Nistari, OS-616 and A13 whereas in control races showed isoforms of DEPO1 and DEPO2 using a Dopamine substrate (Fig.1).

The similar banding pattern and molecular weight was observed in control and LPS- treated midgut samples. By using L-Dopa, LPS induced PO isoforms banding pattern DAPOG3 was observed in viz., Pure Mysore, Nistari, Kollegal Jawan, MY1 (SL), Hosa Mysore and A13. (Fig.2). The midgut from larvae injected with LPS had additional PO isoforms viz., DEPOG3 was present in Pure Mysore, Nistari, Kollegal Jawan, OS-616, MY1 (SL) and HosaMysore. DEPOG1 and DEPOG2 were invariably present in remaining control and LPS-treated races and MW 72, 71 and 46 KDa respectively (Fig.3).

Insects for their better survival generally require congenital environmental conditions in order to keep their normal physiological system [16,17]. Microbial cell wall components such as peptidoglycan, beta-1, 3-glucan, and lipopolysaccharide (LPS) elicit prophenoloxidase (proPO) activation. Microbial components trigger PO in *Ceratitidis capitata* hemocytes [18], silkworm [19,

20]. Melanization requires the activation of proPO to its active form phenoloxidase (PO), a key enzyme that leads to the formation of melanin at wound sites and around intruding microorganisms in the hemolymph had reported by Eleftherianos et al. [21]. Once the ProPo is converted to an active DOPA, and dopamine to melanin [19, 22]. There are two kinds of PO has been identified in silkworm, *Bombyx mori* [19], tobacco hornworm, *M. sexta* [23,24], six in the mosquito, *Anopheles gambiae* [25] and three in the fruit fly, *Drosophila melanogaster* [19]. The above identification correlates PO action with defense function in insects.



Fig.5.31. Native- PAGE of the phenoloxidase using Dopamine substrate in haemolymph of selected silkworm races of *Bombyx mori* (L.). (A) Control,(B) LPS-Treated.

Figure 1: Native-PAGE of the Phenoloxidase using Dopamine substrate in haemolymph of five selected silkworm races of *Bombyx mori* (L.). (A) Control, (B) LPS -treated haemolymph samples.

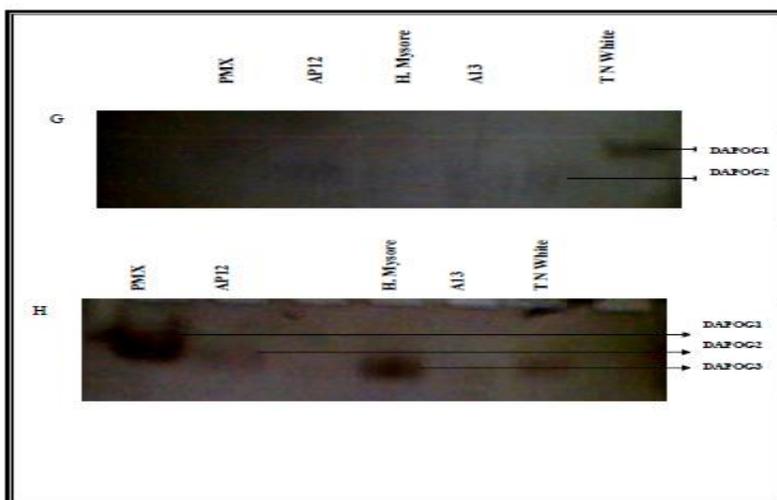


Fig.5.34. Native- PAGE of the phenoloxidase using L-DOPA substrate in midgut of silkworm races of *Bombyx mori* (L.).(G) Control, (H) LPS-Treated.

Figure 2: Native-PAGE of the Phenoloxidase using L-Dopa substrate in midgut of five selected silkworm races of *Bombyx mori* (L.). (G) Control, (H) LPS -treated haemolymph samples.

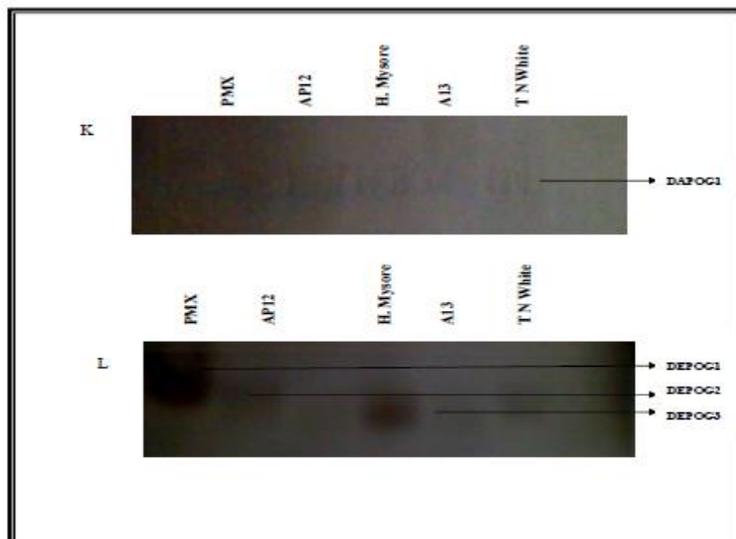


Fig 5.36. Native-PAGE of the phenoloxidase using Dopamine substrate in midgut of silkworm races of *Bombyx mori* (L.). (K) Control, (L) LPS-Treated.

Figure 3: Native-PAGE of the Phenoloxidase using Dopamine substrate in midgut of five silkworm races of *Bombyx mori* (L.). (K) Control, (L) LPS -treated haemolymph samples.

4. Conclusions and Recommendations

The melanization cascade, in which phenoloxidase is the terminal enzyme, appears to play a key role in recognition of and defense against microbial infections in invertebrates. This study focuses phenoloxidase cascade and melanization activity are important for the immune defense towards a highly pathogenic bacterium, *E.coli* endotoxin LPS. The aim of the study is focused to identify the immune defense races using PO as a marker for biotic stress. This report is concluded biotic stress resistant races viz., Pure Mysore, Nistari ,OS-616 , MY1(SL). Hosa Mysore, A13 are moderately tolerant races and remaining races had low level of immune defense against bacterial components. The different isoforms of PO could be used as a marker for index to isolate the better races for increasing crop productivity.

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*Corresponding author.

E-mail address: vishnulifebt@ gmail.com