INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES
IJPAES
VOLUME-2, ISSUE-2 APRIL-JUNE-2012 ISSN 2231-4490
Copy Rights @ 2012 Coden : IJPAES www.ijpaes.com

Received: 15th Mar-2012 *Revised: 19th Mar-2012* *Accepted: 23nd Mar-2012*
Research Article

ROLE OF PROPHENOLOXIDASE (PROPO) IN SILKWORM IMMUNITY- DETERMINATION OF PHENOL OXIDASE (PO) ACTIVITY

G.Sairamalinga Reddy[1]., B.Venkatappa[2]., P.Seshapani[3]., D.Jayasimha Rayalu[4*]., P.B.Janardhan[5]

[1]Department of Microbiology, S.S.B.N Degree & P.G College, Anantapur, AP, INDIA.
[2]Department of Microbiology, Sri Krishnadevaraya University, Anantapur, AP, INDIA.
[3]Department of Microbiology, Sri Venkateswara University, Tirupathi, AP, INDIA.
[4]Department of Biotechnology, DR.Rayalu's Biotech PVT.LTD, Himayat nagar, Hyderabad, AP,INDIA
[5]Department of BioinformatiCane Sugar, Akshaya Biological Corporation, Himayat nagar, Hyderabad, AP, INDIA

*Corresponding Author Adress e-mail: jayasimharayalu@gmail.com

ABSTRACT : A fraction of hemolymph from healthy and bacteria inducted silkworms were collected and total protein content was estimated. Prophenoloxidase in hemolymph of bacteria infected silkworm (Bombyx mori) was partially purified and migrated as two bands in SDS-PAGE system, where as prophenoloxidase band is completely absent in cuticular protein sample of healthy silkworm. Extraction and characterization of pro-phenol oxidase cascade from healthily and bacteria included silkworms was used to determined Phenol oxidase activity. Effect of various sugars on development of phenoloxidase activity was examined. A cane sugar factor (CSF) seems responsible for retention of prophenoloxidase in Cane Sugar-Hemolymph. Microscopic studies revealed that cell clusters or aggregates showed a regular pattern. The results shown that a single protein having molecular mass of 18.5 kDa. These results have clear evidence that upon stimulation by LPS the levels of PEA derived peptide i.e peptide B the 13kDa fragment is significantly increased. Phenoloxidase activity incresed with time when the prophenoloxidase fraction was incubated with the activity cascade (AC) fraction that had been previously treated with Ca^{2+}. Presently results clearly demonstrate that cuticular phenoloxidase is truly is Zymogen form which is activated through a limited proteolosis.

Key words: Prophenoloxidase, Immunity, SDS-PAGE, Hemocytes

INTRODUCTION

Insects possess an innate immune system composed of cellular and humpral immune mechanisms that promptly respond to invasion by microorganisma and other parasites. The cellular immune mechanisms include agglutination of hemocytes. The humoral immune response activated with in hours and generates a diverse set of broad spectrum anti-bacterial proteins and peptides such as cecropins, attacins, defensins and lysozymes [1, 2, 3]. During defense reactions, invariably the foreign organisms are found to be encapsulated and melanized [4]. Melanization is thought to be an important defense reaction of insects. Therefore, melanin and the enzyme responsible for the biosynthesis of phenoloxidase are considered in integral part insect host defense reactions. Phenoloxidase was responsible for melanization and was present in a latent form, which in turn was activated by a proteinaceous activator upon injury. The regulation mechanism of activation of pro-phenol oxidase which is a zymogen of phenoloxidase plays an important role in defense mechanism. Phenoloxidase is present throughout the body of insects including the open circulatory system of hemolymph [5]. The phenoloxidase in insect hemolymph occurs as a proenzyme, prophenoloxidase which can be activated by an activator present inhemolymph and cuticle [6, 7, 8]. Active phenoloxidase is deletarious as it can catalyze the oxidative polymerization of phenols and catechols, but in doing so, it can also polymerize proteins and other macromolecules, posing a potential threat to host.

Hence phenoloxidase is preserved as an inactive proenzyme form prophenoloxidase and is specifically activated proteolytically when it needed. Prophenoloxidase from larval hemolymph of the silkworm is activated by alpha-chemotrypsin ans the reaction is inhibited by spific inhibitorsof the enzyme which is proteolytic in nature [9] prophenoloxidase has been purified extensively from hemolymph of the silkworm larvae and basic properties of protein has been well charecterised [10]. Recently it has been reported that elicitors such as zymosan as wll as cell walls isolated from Gram-negative and Gram-positive bacteria initiate the activation of pro-phenoloxidase of silkworm hemolymph [7, 8]. In this work we demonstrated the effect of different sugars on the phenoloxidase activity.

MATERIALS AND METHODS

Organisms

Silkworms (Bombyx mori) were used for present experiments and reared on mulberry leaves under laboratory conditions. Their life is divided into five stages. Silkworms of stage 4 were used. This stage corresponds to the gametogenesis phase, which is characterized by apoptosis of the salivary or silk glands and empty midgut. More over it is easier to collect hemolymph at this stage of life span.

Estimation of protein from hemocyte free extracts and from hemolymph

A fraction of hemolymph from healthy and bacteria inducted silkworms were collected in equal volume of 10% cold TCA, and kept at 4˚C for 10min. the sample was then centrifuged at 2000 g fro 5min. and the pellet thus obtained was washed twice with cold 5% TCA followed by wash with alcohol ether (3:1) mixture. The pellet was didolved in 0.1N NaOH and used for protein estimation. Total protein content was determined with folin reagent according to method of Lowry et *al (1951)* [11].

Sodium-dodecyl sulphate-poly Acrylamide Gel Electrophotoresis (SDS-PAGE) [12]

SDS-PAGE was performed by using the discontinuos buffer system. The separating gel consisted of 10% (W/V) acrylamide, N,N-Methylene bisacrylamide (sigma,USA) at a concentrations such that the ration of monomer to bis was 30:0.8, 0.0375 M Tris HCL, pH 8.8 and 0.1% SDS. It was chemically polymerized with 0.05% (W/V) ammonium per sulphate (APS) (sigma, USA) and 0.05% (W/V) TEMED (Merck, FRG). The solution was cast into slabs and was overlayed with n-butanol to exclude contract with air. The stacking gel containing 4% W/V acrylamide; 0.12 M Tris-HCL (pH 6.8); 0.1% of SDS; 0.05% (W/V) APS; 0.05% (W/V) TEMED. Samples, 50-200 µg were digested with an equal volume of sample buffer (0.0625 M tris HCL, pH 6.8), 10% (W/V) Glycerol, 5% 2- Meracaptoethanol, 2% SDS and 0.02% Bromophenol blue, by heating in a boiling water bath for 3 min. After cooling the samples along with protein markers (sigma, USA) were loaded into the slots. The samples were stacked and run at 120 V for about 6hrs using 0.025M Tris , 0.192 M Glycine, Buffer pH8.3 containing 0.1% SDS as electrode buffer. After electrophoresis gels were fixed in PBS buffer for 1hr, then the gels were treated with 50% ethanol for 10 minutes each time, and with Na2S2O3 for one minute. Gels were washed with distilled water and then soaked in silver nitrate (AgNO3) solution for ½ hr. further gels were washed with distilled water. Then the gels were washed with fixture mixture II.

Extraction and characterization of pro-phenol oxidase cascade from healthily and bacteria included silkworms [13].

Twenty silkworms larvae of both control and included were dissected in ice-cold acetate buffer (pH 5.2). The integuments were then meticulously separated with a sterile to remove all tissue free cuticles were subsequently rinsed twice in fresh acetate buffer and then blotted with absorbent to remove excess liquid. Finally extracted with acetate buffer for 2hr on broken powered ice. Eight µm of CaCl2 was added to the cuticular extract to make it's final concentration 6mM. the entire mixture was incubated on ice overnight. The incubated mixture was used as prophenol activating enzyme, and subsequently dialyzed against 1 litre of tris buffer overnight at 4˚C.

Assay of phenoloxidase activity

Phenol oxidase activity was determined colorimetrically [14]. The reaction mixture consists of 1 ml of 0.02 M3,4-dihydroxy solution. After incubation at 30˚C for 5 min, the colour intensity was measured by spectrophotometer at 490 nm. One unit of the enzyme was defined as the amount causing increase in absorbance of 0.01 under the above condition. Under these assay conditions activated homogeneous cuticular prophenoloxidase gives a specific activity A520 per 5min for mg of protein.

RESULTS AND DISCUSSION

Aggregation studies

Cell aggregation in normal healthy worms were inducted by 10 mM Ca^{2+} and 1 mM Ca^{2+}supported the aggregation with LPS, however 10mM Mg^{2+} had no effect. These results indicate that LPS inducted aggregation requires Ca^{2+} (Figure I).

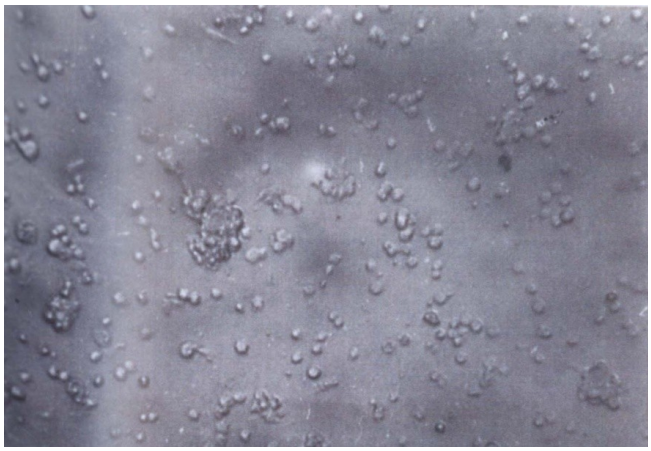

Figure. I: LPS induced hemocyte aggregation (50ug LPS)

Microscopic studies revealed that cell clusters or aggregates showed a regular pattern [15]. Granulocytes always observed in the core of the aggregates and surrounded by plasmocytes (Figure II).

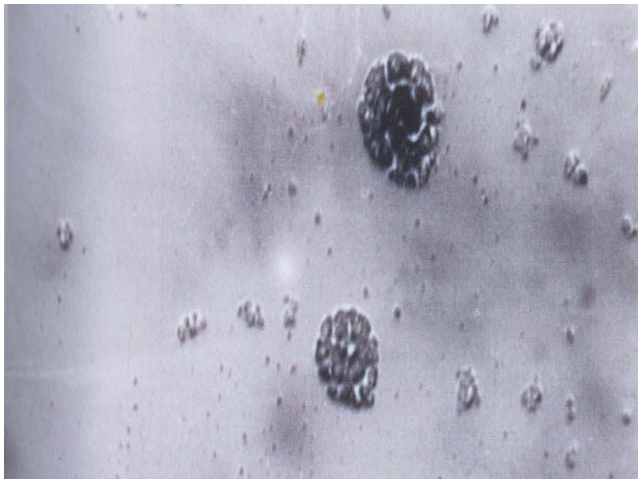

Figure. II: LPS induces hemocyte aggregation (50ug LPS +1mM Ca^{2+})

Because the combination of 1mM Ca^{2+} and LPS inducted best observable aggregation. Further experiments were done using hemocytes collected from pebrine infected silkworms. The effect of infection assayed without Ca^{2+} and LPS. Interestingly hemocytes resulted in a marked decrease in the aggregation. Absence of hemocyte aggregation was due to immune disfunction by pebrine infection (Figure III). Because hemolymph has a cell adhension molecules and recent results from Bettencourt and co-workers (1996) [16] showed that hemolymph is also present in a membrance form on hemocytes. Present results had clearly indicates that the involvement of a hemolymph membrance form on hemocytic aggregation.

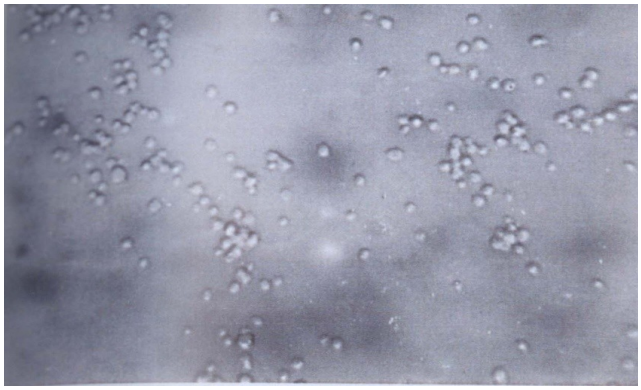

Figure.III: Decrease in the hemocyte aggregation with Pebrine infection

Phenoloxidase activity increased along with the prophenoloxidase activity. Prophenoloxidase activity prophenoloxidase was incubated along with the silkworm cuticular melanin crude extract presently referred as activating cascade (AC) that had been previously treated with Ca^{2+} (Figure IV). Components other than prophenoloxidase in the cuticular prophenoloxidase fraction are not involved in appearance of phenoloxidase activity. these results strongly suggested that prophenol activating factor (PPAF) is a serine proteinase that exists as a zymogen (equal to completment cascade in vertebrate immune system during microbial infection) in the AC extract. In the presence of $Ca^{2+}$the zymogen is slowly activated in the mixture of prophenoloxidase fraction and AC extract when incubated with ice (Figure IV). During experimental system the mixture were held on ice aliquots of 50 µl of the mixture were used to assay phenoloxidase activity which is expressed A520 per 50µl per 5 min at the indicated times (Figure IV) the AC extracts were added to prophenoloxidase activity (50 µl of the mixture) was assayed. If AC extract was not treated with Ca^{2+} no activity appeared. If however, the AC fraction was mixed with prophenoloxidase fraction and Ca^{2+} was added at the same time phenoloxidase fraction and Ca^{2+} was added at the same time phenoloxidase activity gradually appeared (Figure IV).

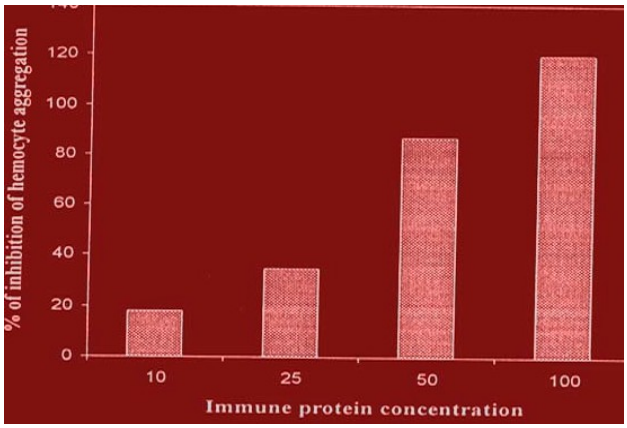

Figure. IV: Effect of Immune protein concentrations on hemocyte aggregation

SDS-PAGE Analysis

Hemocytes were collected at 5 min intervals up to 4h of LPS injection. Electrophoresis was performed on a 10-250% PAGE. antibacterial test on solid medium allowed to detect rapidly in presence of the antibacterial peptides in the hemocyte lysates. The antibacterial activity of the material was assayed by a solid growth inhibition assay using Gram +ve bacteria *M.luteus* and Gram –ve bacteria *E. coli*. Bacteria were grown on luria broth (LB) overnight at 37˚C under agitation. They were then diluted 250µl in 50ml of LB agar placed in petri dishes and stored in 4˚C, 5 to 0.5µl sample were plated on the nutrient agar containing bacteria and incubated at 37˚C overnight. Antibacterial activity was recognized by the lack of grown on the deposit.

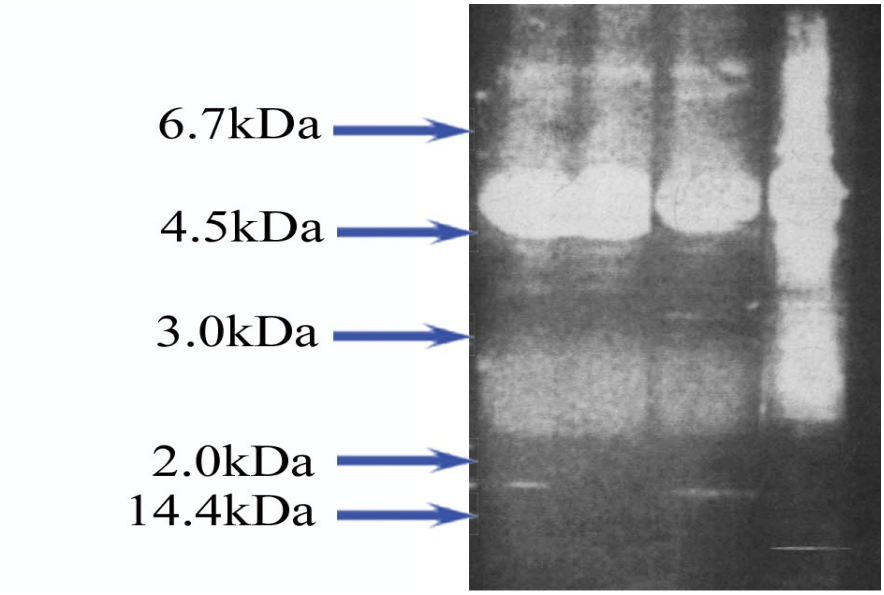

Figure.V: SDS - PAGE Analysis of Silkworm Hemocyte Lysate

Lane-A & C(LPS induced hemocyte lysate), Lane- B (Control). Lane – D (Hemocyte lysate collected 15min post injection).

Analysis of PEA fragments in hemocyte lysate was performed to sustain the hypothesis of the release of peptide B after LPS stimulation. LPS not treated hemocytes lysate treated as control and LPS treated hemocyte lysate were sunjected to electrophoresis. The results shown that a single protein having molecular mass of 18.5 kDa absent control experiment (Figure V). confirming the above results, PEA is consequently present in LPS stimulated hemocytes (Figure V). This molecule correspond to the silkworm hemocyte lysate PEA. We further studied the time course of PEA derived. Secondary molecules release following LPS injection. The hemcytes were collected at different times after injection (15-30Min), fifteen minutes of post-injection PAGE revealed a single protein of 13kDa. This suggest either a quick process of PEA in hemocyte lkysate or a massive release of mature PEA fragment of 13kDa molecule stored in hemocytes (Figure V).

LPS induction studies on Hemocytes

Hemosyte lysate has marked activity (MIC<20 mM) against Gram +ve bacteria *M. Luteus* (100-200nm). The invertebrate immune factor like the mammal material in a concentration dependent manner seemed to contribute to the release of prophenoloxidase or interleukin-1 (1L-1) similar to vertebrate peptide B (Figure. VI). More over the LPS in a dose dependent manner seemed to contribute to the release of peptide B.

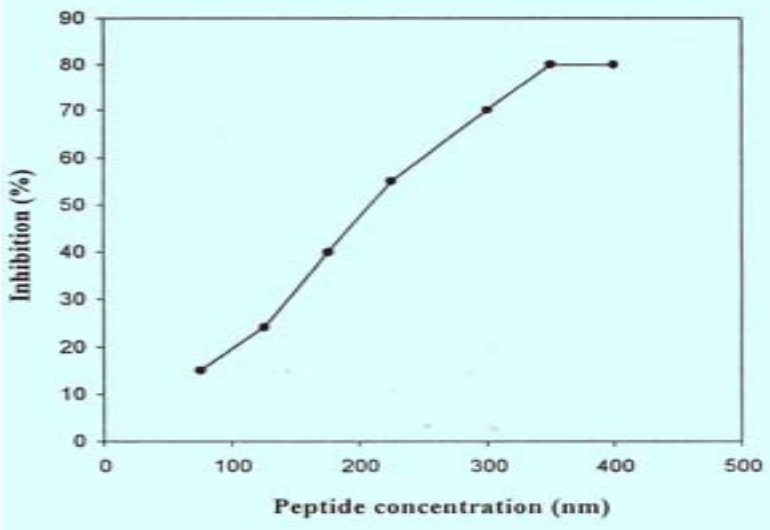

Figure.VI: Antibacterial activity of Peptide-B

Presently we do not know if the effects were related to the LPS or to the injection it self, the results showed that the increase in concentrations of injected LPS provoked a higher release of invertebrate peptide B in hemocyte lysates of silkworms (Figure VII). These results have clear evidence that upon stimulation by LPS the levels of PEA derived peptide i.e peptide B the 13kDa fragment is significantly increased. As the PEA is present in the hemocytes, we suggested two possible processing pathways, an intracellular and/or extracellular processing.

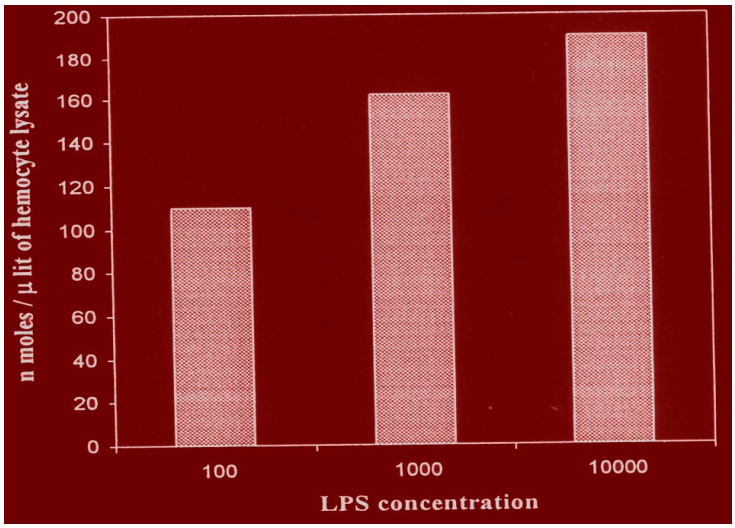

Figure.VII: Effect of LPS dose on the concentration of Peptide- B (15Min after injection)

In part, the significance of these observations is up held by neuropeptide processing. Antibacterial peptides present evolutionary diverse immunocytes, are found with their processing enzymes. When silkworms larvae were inducted with LPS, there were no significant influence on survival and cocoon quality. However, the increase of hemocyte density (Figure. VIII) were clearly restrain and the functional disorder of the fat body was resulted, more over a kind of hemolymph protein was discovered when it disappeared after local injection of LPS. This protein may to be important clue in clarifying the function of unknown hemocytes.

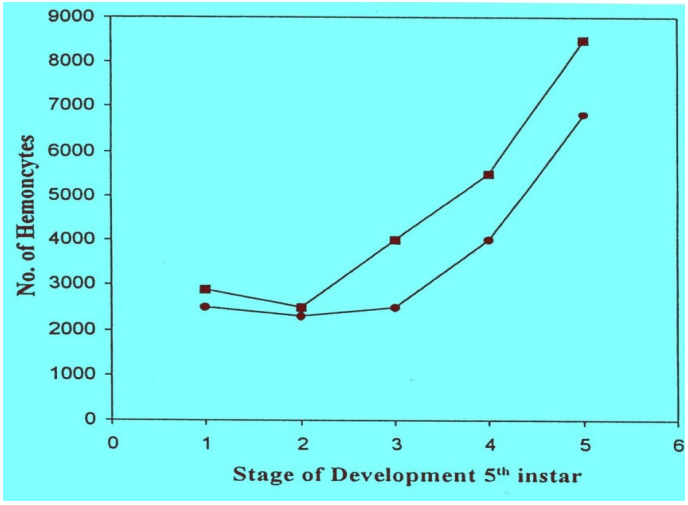

Figure.VIII: Hemocytes densities of Controlled and LPS induced larvae

A: Control B: LPS induced

Action of Various sugars on phenoloxidase in hemolymph

Effect of various sugars on development of phenoloxidase activity was examined (Table I). Surprisingly among sugars tested only Cane Sugar was effective, whereas other mono-and di-saccharides were practically ineffective (Figure. IX). Apparently sucrose itself is not the active principle. Phenoloxidase activity and clotting were not detectable Cane Sugar-Hemolymph. In addition, the total hemocyte count was much decreased when it was examined using hemocytometer. But the effect was less than that of injection of saline containing Cane Sugar. Hemocytes observed in Cane Sugar-Hemolymph were all round shaped.

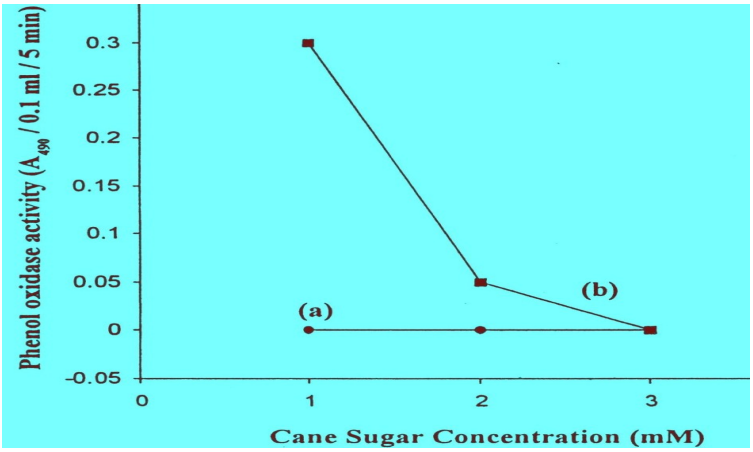

Figure.IX: Suppessing effect of physiological saline containing cane sugar on development of phenol oxidase activity in hemolymph

Physiological saline was injected into haemocoel of silkworm larvae 48 hrs after 4th molt and hemolymph collected

a. PO activity of the haemolymph was assayed immediately after bleeding

b. PO activity after incubation for 20 min at 25^{o}C.

A method for obtaining hemolymph (Cane Sugar- Hemolymph) of the silkworm larve without triggering the activation of prophenoloxidase was described. A cane sugar factor (CSF) seems responsible for retention of prophenoloxidase in Cane Sugar-Hemolymph. CSF was only effective in *vivo.* This method was also applicable to full grown wax-moth larva *(Galleria mellonella)* from which hemolymph with properties similar to Cane Sugar-hemolymph was obtained [17, 18]. Absence of CSF in beet sugar seems to suggest that CFS originates from cane (Table 1). It may be desirable to larvae extracts of cane sugar and other plants to find better source of CSF. Another characteristic feature of Cane Sugar hemolymph is great decrease in total hemocytes.

Table. 1: Effect of various sugars on phenoloxidase in hemolymph

Sugar	PO activity (A490/5min)	Developmental stage of silkworms used hours after 4^{th} moult
Glucose+Fructose	0.193 ± 0.047	42
Mannose	0.214±0.045	150
Maltose	0.290 ± 0.042	66
Trehalose	0.330 ± 0.029	66
Control (without sugar)	0.47 ± 0.33	72

Purification of PGRP

PGRP was purified from 250 ml of larvae silkworms hemolymph [19]. The purification procedure consisted of ammonium sulphate fractionation, column chromatography on peptidogycon-sepharose 4B. As PGRP could not be quantified in hemolymph and the ammonium sulphate fraction, the yield of PGRP in the first steps could not be calculated. In SDS-PAGE under reducing conditions purified as a single band to the position corresponding to that of the 19 kDa polypeptide (Figure. X). Similar molecular mass (16.5 kDa in the SDS-PAGE (Figure. X).

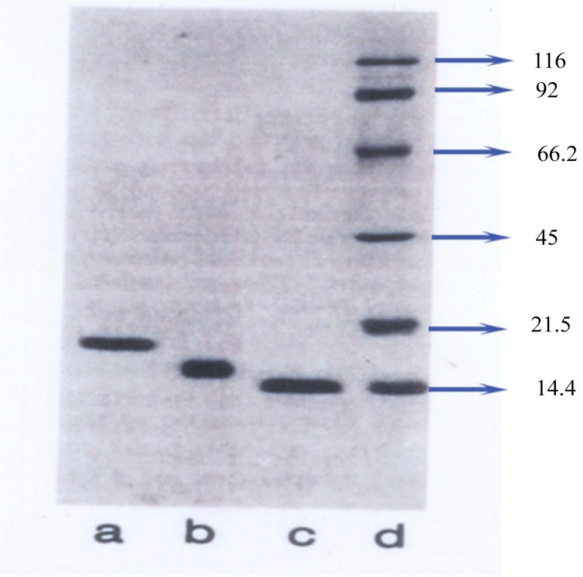

Figure. X: SDS-PAGE Analysis of Peptidoglycon Recognition Protein (PGRP)

Lane –A(PGRP), Lane- B (Insect lysozyme), Lane- C (Egg white lysozyme), Lane-D (Marker proteins)

The restoration of the pro-phenoloxidase cascade to PG in hemolymph- PGRP is shown in (Figure. XI). A decreasing lag period was observed as the concentration of PGRP increased. Once activation of prophenoloxidase is initiated, however the rate of conversion of prophenoloxidase seems to be independent of the amount of PGRP added.

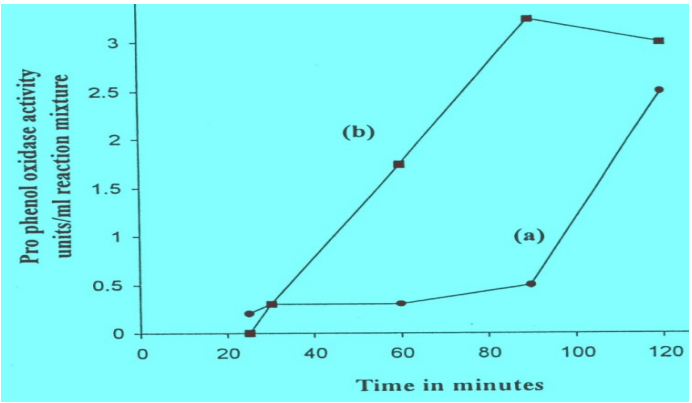

Figure. XI: Effect of supplement peptide glycan recognition protein (PGRP) on activation of the prophenoloxidase cascade

Silkworm hemolymph prophenoloxidase cascade include a PGRP which have specific affinity to PG. these molecules were proposed to trigger the cascade upon binding to their respective ligands. The PGRP preparation was shown to be homogeneous by SDS-PAGE. The molecules are capable of restoring the reactivity of the protein-phenoloxidase. Cascade to PG in hemolynph-PG which is assumed to contain all components of protein-phenoloxidase cascade except for (Figure. XI). These results indicate that the purified protein is PGRP. The assay for PGRP activity by using hemolymph-PG enable us to detect at concentration as low as 90 ng/ml (Figure. XI). However, prophenoxidase in hemoclymph –PG can be activated without PG by unknown factors, in hemolymph of the ammonium sulfate fraction [20]. Such non-specific activation of prophennoloxidase was experienced in the assay of βGRP and the reason for it discussed. To ensure that we assayed PGRP, the effect of given sample on the pro-phenoloxidase cascade in hemolymph-PG was examined both with and without PG and a given sample was judged to contain PGRP only when it could trigger the pro-phenoloxidase cascade in hemolymph PG with, but not without PG.

Phenoloxidase activity incresed with time when the prophenoloxidase fraction was incubated with the activity cascade (AC) fraction that had been previously treated with Ca^{2+}. it was demonstrated that Ca^{2+} was necessary for the AC fraction to acquire the activity to activate prophenoloxidase in the mixture of the fraction (Figure. XII).

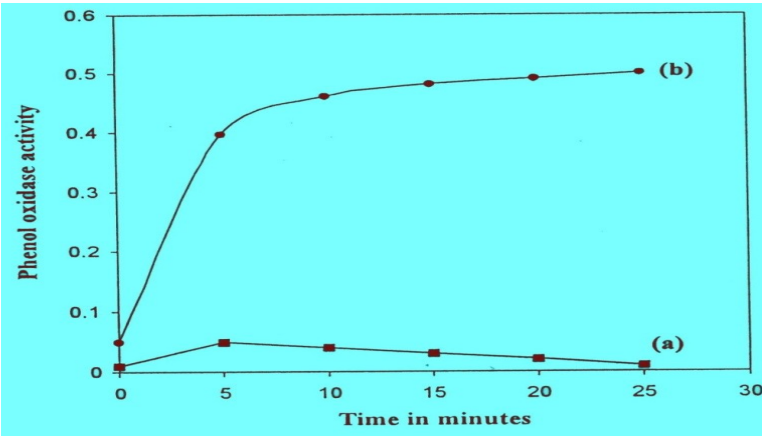

Figure. XII: Assay of Phenol Oxidase Activity

Pheonoloxidase, which is composed of two polypeptides with mobilities in SDS-PAGE corresponding to 71 kDa and 70 kDa (Figure XIII), but did not detect any polypeptides in the activated cascade fraction, indicating the presence of prophenoloxidase only in the prophenoloxidase fraction. The specific activity of phenoloxidase activated at pH 9.0 is very low when compare with pH 6.5 and 7.5 (Table. 2).

Table. 2: Specific activities of phenoloxidase activated at different pH values

PH of activation reaction	Incubation time (min)	Amount of phenoloxidase recovered (µg/protein/ml)	Phenoloxidase activity (units/ml)	Specific activity (Units/µg protein)
6.5	30 60	7.5 7.5	520 560	69.3 74.7
7.5	30 60	12.6 12.5	1000 970	74.9 77.6
9.0	3 6	11.5 10.7	390 380	33.9 33.5

Role of Cuticular and Hemolymph Phenoloxidase (PO) in Innate Immunity

Among the protein extracted cuties with 5% SDS, 3% 2-mercaptoethanol and 5M urea, no polypeptide other than a polypeptide doublet of prophenoloxidase cross reacted with anti-hemolymph prophenonolidase (Figure. XIII). the prophenoloxidase polypeptide were calculated to be 71 kDa and 70 kDa respectively. The mobilities of the prophenoloxidase polypeptide in the pro-phenoloxidase fraction were similar to those in the SDS/urea cuticular extract (Figure. XIII). If the prophenoloxidase fraction is incubated with the fraction in the absence of Ca^{2+}, no difference in mobility could be detected (Figure. XIII). If however the prophenoloxidase fraction was incubated with the Ca^{2+} treated AC fraction as 4-kDa decrease in molecular mass of both polypeptides could be observed (Figure. XIII).

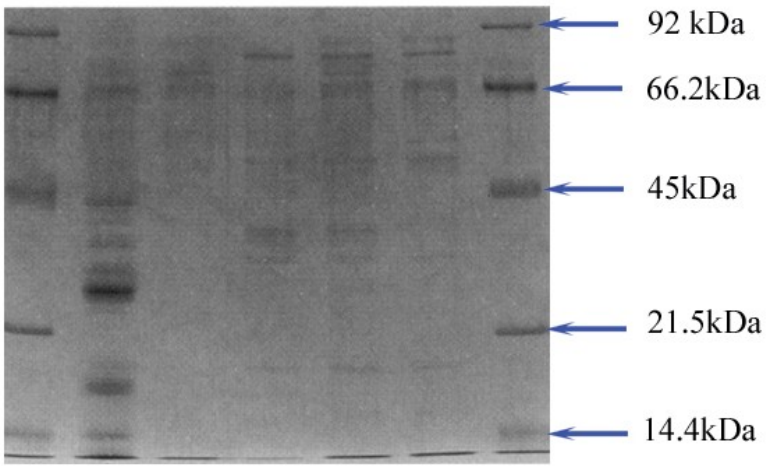

Figure XIII: Detection of pro-phenol oxidase and phenol oxidase by SDS/PAGE

Presently results clearly demonstrate that cuticular phenoloxidase is truly is Zymogen form which is activated through a limited proteolosis by the serine proteinase which is also in a zymogen form. Hence two distinct steps have been elucidated in the activated of prophenoloxidase. Ca^{2+} is necessary for the conversion of pro-PAGE to PPAE, however, we do not know if Ca^{2+} is involved in this specific reaction or if it is involved in cascade events prior to this step. The mechanism by which injury or pathogen invasion set the cuticular into motion remain unknown during our demonstrations.

CONCLUSION

Phenoloxidase is a potentially dangerous enzyme to insects because it oxidizes phenolic compounds to the corresponding quinones. Precursors of granular phenoloxidase and laccase are programmed to be activated at defined times of development. However present results have clear evidence that wound pheloxidase and hemolymph phenoloxidase must be readily activated in response to invasion of microorganisms into the hemocoel. Whatever mechanisms silkworms have exploited, it is obvious that their malfunction would seriouly jeopardize it's survival. For example undesired systemic activation of hemolymph phenoloxidase in silkworms would result in blackening of hemolymph in a matter of seconds and would lead to the death of the silkworms. During our experiements, regulation mechanisms for activation of cuticular phenoloxidase in hemolymph as is suggested by the occurence of many immuno signals affecting the silkworm resistance. Present results along with those of previous studies demonstrate the ubiquity of phenoloxidase throughout the silkworm body (hemolymph and cuticle). The insect tissues literally bathed in or surrounded by this enzyme zymogen and it's activating cascade. Predominant evidence from this results are that prophenoloxidase picks up oxygen from both the vast body surface and tracheal system and transport it through the hemolymph to the tissues.

REFERENCES

[1] Yoshida, H.and M.Ashida. 1986. Microbial activation of two serine enzymes and prophenoloxidase in the plasma fraction of hemolymph of the silkworm, Bombyx mori. Insect.Biochem., 16,3:539-545.

[2] Yoshida, H., M. Ochiai and M. Ashida. 1986. B-1,3-glucan receptor and peptidoglycan receptor are present as separate entities within insect prophenoxidase activating system. Biochem. And Biophys. Resear. Communications,3:1177-1184.

[3] Yoshida, H.,K.Kinoshitag and M. Ashida. 1996. Purification of a peptidoglycan recognition protein from Hemolymph of the silkworm, Bombyx mori. The J.of Biol.Chem., 271,23:13854-13860.

[4] Ochiai, M.,T. Niki and M.Ashida. 1992. Immunocytochemical localization of β-1,3 glucan recognition protein in the silkworm Bombyx mori.Cell issues.Res.268,431-437.

[5] Iwama Reiko and Masaaki Ashida. 1986. Biosynthesis of prophenoloxidase in hemocytes of larval hemolymph of the silkworms, Bombyx mori. Insect.Biochem., 16,3;547-555.

[6] Ashida, M.,R.Iwama and H.Yoshida. 1982. Control and function of the prophenoloxidase activating system. In:Proc.3rd Int.Collog. Invertebrate pathonogy, University of Sussex, Brighton,pp.81-86.

[7] Ashida, M.,and Hiroko. 1.Yamazaki. 1990. Biochemistry of the phenoloxidase system in insects: with apecial reference to its activation. Molting and metamorphosis(ed. By E. Ohnishi and H. Ishizaki). 239-265. Japan sci.soc press,Tokyo/Springer-Verlag,Berlin.

[8] Ashida,M., Ochiai and T.Niki.1988. Immunolocalization of prophenoloxidase among hemocytes of the silkworm,Bombyx mori,20:4,599-610.

[9] Ohinishi, E., K. Dohke and M.Ashida. 1970. Biochemistry of prophenoloxidase specific inhibitions in larval hemolymph of silkworm. Arch.Biochem.Biophys.,139-:143-148.

[10] Dohke,K. 1973. Studies on basic properties of insect hemolymph. Arch.Biochem.Biophys.,157:203-209.

[11] Lowry, O.H., H.J. Rosenbrough, A.Farr and R.J.Randall. 1951. Protein measurement with folin phenol reagent, J.Bio.Chem ., 193:265-275.

[12] Laemmli, U.K. 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T4. Nature (Landon),227:680-685.

[13] Ashida, M., and P.T Brey. 1995. Role of the integument in insect defensis : protein phenol oxidase cascade in the cuticular matrix, proc.Nat.Acad.Sci.92,10698-10702.

[14] Horowitz, N.H.and S.C Shen. 1952. Neurospora tyrokinase.J.Biot.chem .,197:513-510.

[15] Feir,D. 1979.Multiplication of hemocytes. In:Gupta,A.P(Ed.). Insect hemocytes.Cambridge University Press,pp.67-82.

[16] Bettercourt.,R.,H.Lanz Mendoza, and I.Faye.1996. Characterization of insect beads provokoing humoral immune response in Galleria mellonella larvae.j. insect physiol.

[17] Pye, A.E. and W.G.Yendol 1972. Hemocytes containing polyphenoloxidase in Galleria Larvae after injections of bacteria. J.Invertebr.Pathol., 19:166-170.
[18] Pye,A.E. and 1974. Host defense system against insect immunity and parasite resistance. Insect. Biochem ., 8:610-613.
[19] Okada, M. and S Natotri.1983. purification and characterization of an antibacterial protein from hemolymph of sarcophagi peregrine(flesh-fly) larvae. J.Biol.Chem.,260:7174-77.
[20] Brunet, P.C.J.1980.Activation of prophenoloxidase by the activating enzyme of the silkworm Bombyx mori. J.Insect.Biochem.,10:465-500.