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Review:

Antiviral activity in the mulberry silkworm, *Bombyx mori* L.*

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Abstract: The silkworm *Bombyx mori* is exploited both as a powerful biological model system and also as a tool to convert leaf protein into silk. Silkworm larvae often suffer from viral infections causing heavy losses to the economy of the silk industry. Insects exhibit both humoral and cellular immune responses that are effective against various pathogens like bacteria, fungi, protozoa, etc., but no insect immune response is effective against viral infections. To date, no satisfactory reports are available on antiviral immunity of the silkworm. Some efforts have been made by very few workers to identify and characterize the antiviral proteins in the silkworm. In the present article the mode of viral infection, and the activity of certain antiviral proteins involved in silkworm immunity and also in some other insects are discussed. The investigation will be helpful in understanding the molecular aspects of antiviral immunity, disease control and may form the basis for potential use of silkworm in other fields such as medicine.

Key words: Silkworm, Antiviral activity, *Bombyx mori*
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INTRODUCTION

The silkworm *Bombyx mori* has been exploited as a silk producer in the silk industry for thousands of years. Recent success of transgenesis of the silkworm has opened new prospects for this insect species (Tamura *et al*., 2000). The *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a most harmful virus in the sericulture industry, often causing severe economic losses (Ponnuvel *et al*., 2003). It is well known that insects are targets of viruses and it is true especially in the case of lepidopteron insects, where the number of species found to be infected by viruses is twice that compared to other holometabolic orders (Martignoni and Iwai, 1986). The mechanism by which the insect resists viral infections, recognizes infected cells and recruits immune cells to the infective foci or clears infected cells is poorly understood (Popham *et al*., 2004). Insects seemingly lack any adaptive immune responses that operate analogously to the well documented antibody or histocompatibility adaptive immune responses as in vertebrates (Hoffmann, 2003). Apoptosis or programmed cell death is one of the phenomena evolved by certain vertebrates and invertebrates lacking humoral immunity to function as antiviral defense mechanism (Narayan, 2004). This mechanism is a controlled biochemical pathway distinguishable from cell necrosis by characteristics that include cellular shrinkage, membrane blebbing, chromatin condensation, apoptotic body formation and fragmentation (Wyllie *et al*., 1980).

Insect immunity plays an important role in the interaction between the host and pathogen as a part of survival strategy including physical blockades such as cuticle and peritrophic matrix, epithelial barriers, protease cascades leading to coagulation and melanization, cellular responses such as phagocytosis and encapsulation and also the production of certain

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antimicrobial peptides (Lavine and Strand, 2002; Levashina *et al*., 2001; Ligoxygakis, 2002; Lehane, 1997; Lehane *et al*., 1997; 2004; Meister, 2003; Tzou *et al*., 2000; Vernick *et al*., 1995). Studies pertaining to defensive mechanisms of insects against various pathogens like bacteria, fungus and protozoa are well documented; but reports on antiviral mechanisms are scanty. It is truly surprising to note that among the thousands of published articles addressing insect baculoviruses as efficient recombinant protein expression systems, there are virtually no studies dealing with the mechanisms of antiviral responses elicited by the insect host cells. This means that special attention must be focused on this area to determine the genes and proteins involved in conferring resistance to modulate their expression in genetically modified cell lines and silkworms (Prudhomme and Couble, 2002). Apart from this, the omnipresence of insects in any type of ecological system, from waterway to extremely septic environments, has stimulated research into their potential use as therapeutic agents (Bulet *et al*., 1999; Chernysh *et al*., 2002). The haemolymph of tobacco budworm, *Heliothis virescenes* has antiviral activity against seven DNA and RNA viruses including HIV-1 that can infect humans. This antiviral compound is an N-myristolyated peptide having molecular weight of 916 Da and containing six amino acids (Ourth, 2004). Hiraki *et al*.(2000) showed the presence of antiviral protein in silkworm faeces was effective against HVJ virus. The purification and characterization of an antiviral agent produced by insect larva is an important discovery of immense medical importance (Ourth, 2004).

Proper understanding of host-pathogen interaction, defensive mechanisms evolved in the host body in response to infection, anti-defensive/immunosuppression molecules released by pathogen to suppress host immunity is necessary before stepping into other aspects like disease control. In this context, it is important to study on recent developments in the antiviral and antimicrobial proteins in insects. In this direction, we initiated studies and obtained information on the proteins involved in antiviral mechanisms against BmNPV in the silkworm and other insects. We hope that knowledge of the silkworm's immune response to viral particles not only helps in the control of viral diseases in the economically important insect like silkworm, but also helps to use silkworm as a bioreactor for the development of antiviral agents important in human health and welfare.

MODE OF INFECTION

Baculoviridae is a family of enveloped double stranded DNA viruses infecting arthropoda, and other insects, especially lepidoptera. Individual baculoviruses typically have very narrow host range, although AcMNPV infects nearly 30 lepidopteron species. BmNPV is a species affecting only the silkworm. The virus particles require two phenotypically different but genetically identical virions to complete their life cycles, both forms have different role during pathogenesis (Engelhard and Volkman, 1995; Maeda, 1989; Nakazawa *et al*., 2004). The virus particles are embedded in proteinaceous occlusions which are released into the larval midgut by the combined action of alkaline gut pH and proteases. Infection begins when the envelops of occlusion derived virus (ODV) fuse with the microvillar membrane of mature or differentiating columnar cells (Engelhard and Volkman, 1995; Granados and Lawler, 1981; Hortan and Burand, 1993). Infected midgut cells produce virions primarily as single nucleocapsids through the basal plasma membrane. This budded virus (BV) thereby gains an envelope studded with GP-64, a viral encoded glycoprotein which is important for the infection of neighboring host cells and tissues (Monsma *et al*., 1996). Cells of the insect tracheal system are the important targets of BV, and their infection is critical for the rapid spread of the virus because they provide access to larval tissues surrounded by basal laminar barriers (Engelhard *et al*., 1994; Washburn *et al*., 1995). By the end of the infection cycle, the body of the lepidopteron larvae is liquefied and transformed into millions of new occlusion bodies that spread into the surrounding environment.

PROTEINS INVOLVED IN ANTIVIRAL MECHANISMS OF SILKWORM AND OTHER INSECTS

As the primary route/major route of infection is through the food, there must be some antiviral mechanism/substances existing in the gut juice of the

caterpillar. The presence of such antiviral substances was observed by some of the earlier workers (Aizawa, 1962; Funakoshi and Aizawa, 1989). A number of antibacterial and antiviral substances have been identified and isolated from the haemolymph and intestinal fluids of silkworm.

Hayashiya and Nishida (1968; 1976) and Hayashiya and Matsubara (1971) reported the red fluorescent proteins (RFPs) could inactivate the BmNPV. RFP is a conjugated protein bearing a chromophore, a kind of bile pigment showing two absorbance peaks (at 280 and 605 nm wave length) emiting fluorescence at 335 and 620 nm after excitation at 280 and 550 nm, respectively. Investigation of RFPs physiological activities revealed that it had antiviral effect against BmNPV and Galleria NPV.

In the synthesis of this protein, chlorophyll-a of mulberry leaves is first converted into chlorollide-a by chlorophyllase under the action of light and is further synthesized in the midgut cells and released into the midgut to form RFP. Chlorophyllase enzyme catalyses the hydrolytic cleavage of the phytol moiety from chlorophyllide setting free phytol and chlorophyllide. The prophyrine ring of chlorophyllide breaks out from chlorophyllide to change into bile pigment. For this reaction light and oxygen are most necessary. Both chlorophyll-a and chlorophyllase are essential for RFP synthesis. Chlorophyllide-a pigment can be conjugated unspecifically with more than one midgut protein, with the ingested chlorophyll merely providing a pigment moiety for RFP biosynthesis. Thus, RFP as a whole may be regarded as a group of midgut proteins emitting red fluorescence when conjugated with chlorophyll from mulberry leaves in the presence of chlorphyllase. Experiments revealed that RFP is detected only in the digestive juice of the mulberry raised larvae but not found in the juice of larvae reared on artificial diet without mulberry leaf powder. It was observed that silkworm larvae reared continuously in the dark are more susceptible to peroral infection with NPV than those reared continuously in environment with light. The increased susceptibility may be due to absence or lower production of RFP in the larvae reared in the dark.

Although the exact mechanism of the antiviral action of RFP is still unknown, it is believed that it destroys the nucleocapsid of NPV or blocks the multiplication of NPV or agglutinates the virus and is excreted along with faeces.

Further efforts are needed to obtain know on the exact mechanism of this protein inhibiting the multiplication of BmNPV, identifying the genes responsible for production of RFP. Possibilities of increasing the production of RFP either naturally or by fortifying adjuvants along with mulberry leaves. If this could be done, the silk industry could be still more profitable and sustainable (Jayaprakash and Rachappa, 2000).

Thereafter, except for some recent studies on serine proteases and lipases (Nakazawa *et al*., 2004; Ponnuvel *et al*., 2003), the molecular characteristics of this protein have not determined so far and the DNA sequence of this protein is also not available in the gene bank. Lipases are likely to contribute immune defenses, conceivably acting directly against invading microorganisms. Bmlipase-1, a lipase purified from the digestive juice of *B*. *mori* larvae proved to have a strong antiviral activity against BmNPV and showed 56% homology with *Drosophila melanogaster* lipase and 21% homology with human lipase. It has also been confirmed that the Bmlipase-1 gene is expressed only in the midgut tissue, but not in other tissues. When the fifth instar larvae of silkworm were orally inoculated with pre-treated BmNPV-ODV (ODV incubated with Bmlipase); the larvae showed resistance to viral infection and successfully entered the pupal stage, thereby indicating the suppression of viral proliferation by midgut Bmlipase1 (Ponnuvel *et al*., 2003). In *Drosophila melanogaster*, four lipase genes were found to be induced upon immune challenge (de Gregorio *et al*., 2002). The inhibition of equine and porcine rotavirus by alpha-amylase and lipase also was observed.

Serine proteases are among the group of proteins that regulate several invertebrate defense responses including haemolymph coagulation, antimicrobial peptide synthesis and melanization of pathogen surfaces (Gorman and Paskewitz, 2001). Nakazawa *et al*.(2004) showed that the presence of serine protease in the digestive juice of silkworm larvae, has strong antiviral activity against BmNPV. The molecular mass and partial N-terminal sequence was also determined. Since, the deduced amino acid sequence of the cDNA showed 94% homology with *B*. *mori* serine protease, they designated it as BmSP-2, and reported that there may be about five serine protease isoforms

including BmSP-2. Recently, in our laboratory we cloned and sequenced two anti-viral proteins namely Bmlipase and BmSP-2 from the midgut tissue of Chinese silkworm and wild silkworm, *Bombyx mandarina* (unpublished data).

The haemolymph of mosquito, *Anopheles gambiae* also contains five serine proteases, which play an important role in insect immunity (Gorman and Paskewitz, 2001). Among them sp22D was found to be the largest protease with potential pathogen binding domains that is expressed in the midgut epithelium, fat body and haemocytes. sp14A, sp14D1 and sp14D2 are clip domain serine proteases that are similar to enzymes with presumed roles in melanization.

The first step in any immune response is the recognition of an invading organism as foreign. Once such recognition has taken place, a protective response involving blood cells or soluble plasma proteins (Yu *et al*., 2002) may be triggered. Innate immune system in both mammals and arthropods utilize proteins known as pattern recognition proteins or receptors, which perform surveillance function by binding to molecules common to the groups of microorganisms (Hoffmann *et al*., 1999; Hoffmann, 2003; Janeway, 1989). Pattern recognition proteins lack the binding specificity of antibodies, and instead function by binding to classes of polysaccharides, such as lipopolysaccharide (LPS), lipoteichoic acid, peptidoglycan and β-1,3-glucans, present on the surface of bacteria and fungi. This type of recognition leads to rapid and broad responses to infection, comprising typical innate immune systems (Yu *et al*., 2002).

Lepidopteron larvae resist baculovirus infection by selective apoptosis of infected cells from the midgut epithelial cells and by sloughing off infected cells from the midgut cells. Even then the virions can successfully enter the host body. At that stage, how does the host defense mechanism operate to control the multiplication of virions? What are all the genes and proteins involved in the antiviral mechanism? Answers to these questions remained obscure.

As a consequence of the increased viral load, lepidopteron insects have evolved both immune responses and physiological counter measures against viral infections (Terenius, 2004). The positive role of haemocytes in clearing microorganisms from the haemolymph of insects by forming melanotic capsules is very clear (Ashida and Brey, 1997), but the haemocyte role does not seem to be uniform among different lepidopteran species. The haemocytes can have different roles from actively spreading the virus to avoiding being infected, to clear virus from the haemolyph (Terenius, 2004). Some studies on baculovirus ecology and physiology explain the possibility of the antiviral defense mechanism by hemolin in this insect order (Hirai *et al*., 2004). Hemolin is the only insect member of the immunoglobulin (Ig) super family reported to be up-regulated during an immune response. It is composed of four immunoglobulin domains. A number of reports are available suggesting the presence of hemolin in the midgut and haemolymph of the insects, especially in lepidopteron insects. Since, hemolin binds to haemocytes and bacteria, it can be assumed that it functions as pathogen recognition protein and also as an opsonin, thus increasing the efficiency of phagocytosis (Kanost and Zhao, 1996). However, whether hemolin can mediate the binding between haemocytes and virus remains an open question (Terenius, 2004).

It has been reported that synthesis of this protein is strongly induced by bacterial challenge in *Hylophora cecropia* and *Manduca sexta*. Yu and Kansot (1999) illustrated the developmental expression of hemolin in the bacterial challenged *Manduca sexta*; they found low levels of hemolin in the immature larvae, but it subsequently increased prior to pupal stage and remained high throughout the pupal and adult stages and also in eggs laid, and persisted throughout the embryonic development. The appearance of hemolin mRNA in fat body and midgut at the beginning of the wandering stages of this insect correlated with the presence of hemolin in the hemolymph and midgut lumen. The cDNA isolated from the fat body of adult *M. sexta* had the same sequences as those obtained from larval libraries thus suggesting the developmental regulation of this protein by the same hemolin gene throughout the life-cycle of an insect and also expression of this particular gene at the time of microbial infection. In diapausing pupae of *Hylaphora cecropia*, the hemolin gene is expressed in fat body cells and in haemocytes (Roxstrom-Lindquist *et al*., 2002). The gypsy moth *Lymantria dispar* is known to have a 55 kDa hemolin belonging to the immunoglobulin superfamily. The

mRNA levels of hemolin increased at the time of diapause initiation, remained constant throughout this period and dropped in late diapause, thus offering protection to the moth against invading pathogens (Lee *et al*., 2002) during the crucial stage of the life-cycle. The above reports suggest the induction of hemolin in the haemolymph of lepidopteran insects as a response to bacterial infection. The induction of hemolin in baculovirus injected pupa of Chinese oak silk moth *Antheraea pernyi* was proved by Hirai *et al*.(2004). Does it play an active role in antiviral immunity? If so, what are all the underlying mechanisms by which it exhibits antiviral activity? These are all questions to be answered. To our knowledge there is no single report showing the antiviral activity of hemolin in silkworm.

PKR is a double stranded RNA-activated protein kinase, which in recent years has been extensively studied, with large body of evidence being accumulated concerning its expression, interaction with regulatory RNA and protein molecules, and modes of activation and inhibition. PKR has been shown to play various important roles in the regulation of translation, transcription and signal transduction pathways through its ability to phosphorylate protein synthesis initiation factor eIF2, I-KappaB and other substrates. Expression studies involving both the wild type protein and dominant negative mutants of PKR have established roles for the enzyme in the antiviral effects of IFNs, in the responses of uninfected cells to physiological stresses, and in cell growth regulation (Clemens and Elia, 1997). The possible role of PKR functioning as a tumor suppressor and inducer of apoptosis suggests the importance of IFN-regulated protein kinase in the control of cell proliferation and transformation. *B*. *mori* cDNA showed high similarity in its kinase domain to the vertebrate anti-viral kinase (PKR), it was isolated, cloned and sequenced from silkworm. The active role of this protein in the silkworm still remains to be established (Prudhomme and Couble, 2002). The increased levels of PKR activity are known to induce the apoptosis in response to viral infections, thus suggesting the positive role of this protein in the induction of apoptosis. But the baculovirus has developed countermeasures to combat the anti-viral defense mechanism of the host by synthesizing antiapoptotic proteins like p35 and inhibitors of apoptosis (IAP), so as to prevent cell death induced by the insect cell apoptotic machanism (Clem *et al*., 1991; Prudhomme and Couble, 2002). The phenoloxidase in insects also exhibits anti-microbial activity; and produces melanin as an end product. Melanization is a process used for healing wounds and encapsulation of micro-organisms. The cuticle is considered as first line of defense that is indispensable to safeguard environmental factors such as drought and infectious microbes (Asano and Ashida, 2001). Though the possible role of cuticular pro-phenoloxidase as a recognition system for bacteria and fungi has been explained by several workers, participation of this protein in insect immunity is not clear. In 2001, the isoforms of cuticular and haemolymph pro-phenoloxidase in silkworm were purified and characterized by Asano and Ashida (2001). Terenius (2004) illustrated the depletion of phenoloxidase in the *H*. *cecropia* pupae after injection with dshemolin and *E*. *clocae* β12 and he concluded that the interference in mRNA production of hemolin by using dsRNA leads to reduction in phenoloxidase activity.

The feces of silkworm larvae also possess an antiviral substance L4-1, which is active against HVJ (Sendai virus)-LLC-MK2 cell system. The antiviral activity of L4-1 depends on light irradiation and its activity can be inhibited by sodium sulphite and anaerobic conditions. It was shown that the antiviral activity is due to damage to viral proteins caused by active oxygen species produced from L4-1 (Hiraki *et al*., 2000). Lim *et al*.(2002) stressed the utilization of chlorophyll derivatives (CpD) from silkworm excreta in photodynamic antimicrobial chemotherapy (PACT) in order to avoid the viral infections through blood transfusion. In their study, they used vesicular stomatitis virus (VSV), as target organism to demonstrate the antiviral effect of chlorophyll CpD-PACT. Further, they observed inhibition of viral RNA synthesis and decrease in M protein in the host cells inoculated with CpD-PACT treated virus and suggested CpD is an efficient photodynamic antiviral agent. Some of the dipteran insects were shown to produce antimicrobial peptides called alloferons, a group of peptides which possess antiviral and antmicrobial capabilities thus indicating the immunomodulatory properties (Chernysh *et al*., 2002). The haemolymph of tobacco budworm, *Heliothis virescenes* also possess antiviral activity against seven DNA and RNA1 viruses that can infect humans including HIV-1. This

antiviral compound is an N-myristolyated peptide having molecular weight of 916 Da and containing six amino acids (Ourth, 2004). So far no such studies were made on domesticated insect *B. mori*, and efforts in this direction open new vistas of research in silkworm which will not only improve the silk industry economy but also human health and welfare.

CONCLUSION

The mulberry silkworm, domesticated and mass reared for several centuries, presumably has weakened immune system which has made the insect highly vulnerable to bacterial and viral infections. Information on the organization and function of the immune system in the silkworm in general and with reference to viral infections in particular is scanty. There are a few recent reports on the presence of antiviral proteins, against some DNA and RNA viruses, which strongly suggests the presence of a functional antiviral immune system in the silkworm. A number of mechanisms operate at different levels, from the site of origin of infection viz midgut through the medium of transport viz hemolymph to the peripheral tissues where virus proliferates, stalling the initiation, propagation and multiplication of the virus. There are a few scanty reports on the host-parasite interaction at each of the three levels but detailed investigations are required before stepping into disease control and also other aspects such as utilization of silkworm as a bioreactor to produce antiviral proteins of interest and having application in human health and welfare.

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