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Review Article

KEY BIOCHEMICAL MARKERS IN SILKWORMS CHALLENGED WITH IMMUNO ELICITORS AND THEIR ASSOCIATION IN GENETIC RESISTANCE FOR SURVIVAL

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ABSTRACT
Sericigenous insects like silkworm possess an effective immune system against pathogens. The knowledge on the immune system of these insects implicating the mechanism of resistance to diseases is mainly attributable to the presence of key biomolecules such as an inducible proteins *viz*., cecropin, attacin, lebocin, moricin and isoforms of enzymes *viz*., carboxyl esterase and prophenoloxidase which are reproducible qualitative biochemical markers would favor us in enhancing our theoretical level on the mechanism of immunity. Studies on these key biomolecules in silkworms challenged with immuno elicitor *viz*., lipopolysaccharide (LPS) as a function of immunity and survival ability of the silkworms revealed the mode of cellular and humoral functions combating the diseases in nature and these factors can be used by researchers or breeders to identify the appropriate hardy genetic material from the germplasm stock for further breeding work. In this review article an attempt has been made to delineate those key biomolelcules which are qualitatively reproducible and that support survival of the silkworms of *Bombyx mori* (L). These key factors may be considered to identify the genetic resistance of various silkworm races/breeds of *Bombyx mori* and documentation of hardy silkworm breeds for field exploitation in different climatic conditions of the tropical zones of the country.

KEYWORDS: Biochemical marker, Silkworm, immune elicitors, genetic resistance etc.

INTRODUCTION

Conservation of economically important sericigenous insects in varied agro climatic conditions are imperative to preserve their genetic potential and for survival to ensure the maintenance of the genetic material and taping of the yield potentials by the commercial exploiters in the silk industry. The survival character of sericigenous insect *Bombyx mori* (L) varies because several silkworm races/breeds were developed by the breeders suiting to the specific need of varied agro climatic conditions prevailing in the tropical zones of India for their better survival and higher crop productivity. Such developed silkworm races /breeds are many as much as 450 different races/breeds which are being conserved and maintained at Central Sericultural Germplasm Resources Centre, India for utility of them by researchers/breeders in the country (Thangavelu *et al*., 1997 and 2001). Conservationists are interested to maintain these races/breeds for harnessing their genetic potentials for higher silk production. However, the breeders always look for high surviving breeds apart from the good economic characters because the effective rate of rearing of these races/breeds fetch higher cocoon yield and in turn better remuneration from the market. India is a vast country with varying climatic conditions in different agro-climatic zones. The precipitation rate, temperature and humidity vary from season to season and zone to zone. Among different zones, the tropical regions experience the highest temperature as well as humidity (Sengupta *et al*., 1997). These tropical regions support the growth of polyvoltine silkworm breeds/races because of their hardy nature and robustness in their hardy survival. There are other breeds *viz*., bivoltine which are temperate in origin being well exploited in the tropical region for their better genetic potential of higher productivity and moderate survivability in the tropical zones. Therefore, breeding of silkworms from these polyvoltine and bivoltine germplasm breeds since long has been aimed towards evolving superior breeds either by means of selection alone or by out crossing and back crossing followed by selection and subsequent generations to get a higher survival and higher productivity a combination which is found lacking either in polyvoltine and bivoltine breeds (Raju and krishnamoorthy, 1993; Das *et al*., 1998; Das, 2001).

Introduction of polyvoltine blood, which is genetically hardy as far as disease resistance concerned into less resistant bivoltine breeds for developing hardy bivoltine breeds, is practiced more in China (Das 2001; Das *et al*., 2005 a b; Chandrasekariha and Ramesh Babu, 2003). To know the genetic potential for survival of these breeds, an approach of biochemical analysis of immuno competence of them would throw a better light on the role of key biomolecules associated with survival factors which support their ability to resist diseases in the prevailing climatic conditions. The isozymes and total induced proteins were used to identify silkworm breeds, possessing

thermo tolerant and disease resistant characters (Ladizisky, 1975 and Somasundaram *et al*., 2009).

In this context, the key biochemical factors that play a vital role in successful attempt of any breeding programme resulting in selection of suitable parents for choosing them as suitable breeding germplasm stocks. The suitable breeds having specific characteristics of thermo tolerance as evidenced by higher survival rate are available in polyvoltine breeds which are moderate in their productivity character by virtue of their natural genetic hardiness on account of their habitation in the tropical origin, whereas the bivoltine breeds are less genetically hardy coupled with high productive character due to temperate climate supports (Sengupta *et al*., 1997 and Suresh Kumar *et al*., 2004).

In view of the above, there is a need for introgression of the special character of genetic hardiness from the multivoltine breeds into bivoltine. Therefore, there is a need to identify some of the biochemical parameters associated with genetic resistance so that such breeds having higher immune competence genetic resistance for higher survival may help to choose silkworm accessions for breeding to evolve bivoltine pure breed/hybrids introgressed with hardy characters from the polyvoltine breeds for commercial exploitation. In this background, the importance of biochemical parameters which are required to understand the role of key biomolecules associated with insect immunity, numerous studies were carried out to know the biochemical mechanisms *viz*., antibacterial proteins and induced isoforms of many enzymes playing key role in imparting immunity in insects (Yamakawa and Tanaka, 1999).

One among such biochemical parameters is the role of isozymes. There are reports to relate the importance of esterase isozymes associated with higher survival ability of polyvoltine silkworm breeds, which are genetically hardy in their survival in any harsh agro climatic conditions (Moorthy *et al*., 2007). This survival character as found in these polyvoltine silkworm breeds is not high in bivoltine silkworm breeds, which are other highly productive in economic characters. Therefore, there is always many research attempts in the silk industry to introgress this specific character from the polyvoltine silkworm breeds into bivoltine breeds in order to stabilize the survival ability of the latter for its robustness and productivity to augment higher silk productivity in the tropical countries. Central Sericultural Germplasm Resources Centre, India is the nodal centre for conservation of different silkworm races/breeds and its food mulberry plants belonging to different species of morus family for diversity in their respective genetic resources. The silkworm breeds of *Bombyx mori* (L) being poikilothermic animals behave in their thermal acclimation to different agro climatic areas and in accordance with that their quantitative and qualitative traits expression vary deciding the crop success in a given environment. Therefore, innate immunity in silkworms supporting higher survival rate need to be studied to know the involvement of antigen-antibody mechanisms especially against bacterial infection through the immuno elicitor and thereby conferring necessary resistance against pathogenicity. To date, many antibacterial proteins have been isolated from different species of insects (Hara and Yamakawa, 1995) and classified into 5 major groups *viz.*, cecropins, insect's defensins, and attacins like proteins, proline rich peptides and lysozymes (Boman and Hultmark, 1987; Hultmark, 1993)

Cecropins are thought to be primarily responsible for the antibacterial activity in some insects since they show antibacterial activity against many kinds of Gram +ve and Gram -ve bacteria. Antibacterial proteins are important factors involved in humoral defense reaction in insect immunity. Insect antibacterial proteins are rapidly synthesized in specific tissues and secreted into the hemolymph after bacterial infection or injury. In silkworm *Bombyx mori* an immune mechanism such as phagocytosis, encapsulation, carboxyl esterase and prophenoloxidase cascades and their synthesis as antibacterial proteins effectively engaged in defense reactions against invading pathogens/immuno elicitors were investigated and documented (Shiotsuki and Kato, 1999). Apart from earlier reports on antibacterial proteins like cecropins, attacins and lysozymes three novel proteins such as lebocin, moricin and haemocytein have been isolated from *Bombyx mori* and are the immune system of insects and in silkworm comprises a complex network of cells and molecules that protect the organism from infection. Insects have specific defense system to protect themselves from bacterial invasion and are called innate immunity (Boman, 2000 & Zasloff, 2002). Invasion of bacteria induces a variety of antibacterial polypeptides and triggers protenaceous interactions (Gillepie *et al*., 1997). Atttacin, cecropin, lebocin and moricin are antibacterial polypeptides induced in the silkworm are induced when the silkworm larvae are injected with lipopolysaccharide (LPS) from Escherichia coli (Russell and Dunn, 1996; Yamakawa and Tanaka, 1999). Knowledge of insect immunity is still very limited in comparison with genomic knowledge of it (Wang *et al.*, 2004). Insect produces a battery of proteins when stimulated by microbial infection. (Boman, 1991). Antimicrobial molecules such as cecropin, which destroys the bacterial membrane by disturbing its structure (Boman, 1995) and lysozyme, which degrades the bacterial cell wall (Daffre *et al.*, 1994), were among the first identified. Later, other types of microbe-inducible proteins, *e.g*., nonself –recognition molecules such as hemolin (Betterncourt *et al*., 1997) and proteases (Dimopolos *et al*., 1997) were identified. Many bacterially inducible proteins have also been reported (Hughes *et al*., 1983; Kang *et al*., 1996). These findings indicate that the immune response induced by bacterial infection/immuno elicitor LPS involves the production of both antibacterial molecules inducible proteins and different isoforms of enzymes with function not yet fully understood. The function of Phenoloxidase (PO) is thought to be a part of the recognition system of foreignness in insect immunity and PO is a key component of the primary immune response in arthropods.

The most important character that determines the commercial success of any silkworm breed is its tolerance to diseases under adverse conditions is characterized by inherent immune responses of the breed. Immune response activities are responsible for protecting the insects from pathogenic and non-pathogenic bacteria. Swiftness of

clearance of ingested bacteria is determined by the combined action of cellular and humoral defenses of insects.

Immune responses of insects can be studied by following the pattern of colonization of bacteria in the haemolymph *in vivo.* Studies with *Hylophora cecropia* showed that the injected bacteria, *Escherichia coli* could be cleared at around 48 h after post injection (Faye *et al*., 1975; Flyg *et al*., 1987; Kaaya *et al*., 1987). Induction kinetics of antibacterial activity showed that after the initial lag period, immune system is activated which the bacterial multiplication in check keeps leading to clearance of inoculated bacteria (Faye *et al*., 1978). Further studies are lacking on immune response parameters like changes in prophenol oxidase activity, Cecropin and Lysozyme units to ascertain the immune competence of these breeds (Thangamalar *et al*., 2010). In silkworm *Bombyx mori*, innate immune mechanisms such as phagocytosis, cellular encapsulation, prophenol oxidase cascade, and synthesis of antimicrobial proteins with antimicrobial activity are effectively engaged in defense reaction against invading pathogens.

Cellular reactions involve phagocytosis, nodule formation and encapsulation by plasmatocytes and granulocytes. Antimicrobial proteins appear to be ubiquitous and multi-components of the innate immune mechanisms existing in *B. mori.* Several immune proteins have been isolated from *B. mori* and their amino acid sequences determined. Apart from the earlier reported antibacterial proteins like cecropin, attacin and lysozyme, three novel proteins such as lebocin, moricin and hemocytin were isolated from *B. mori* (Russell and Dunn, 1996: Yamakawa and Tanaka, 1999).

The antibacterial mechanisms of lebocin and moricin have been analyzed and their ability to form ion channels in bacterial membranes shows their important role in defense against bacterial infection. Interestingly, hemocytin plays a dual role in immune mechanism against bacterial infection as well as in metamorphosis.

In general, the insect immunity consists of cellular and humoral reactions. The biochemical study that is needed to link the immuno competence through reproducible qualitative markers involve the investigation of humoral reactions such as activation of enzyme cascades like prophenol oxidase and stress related enzyme esterase cascades and induction of immune proteins such as lysozymes, lectins, antibacterial proteins and antifungal proteins. Both immune reactions work in concert to prevent insects from acquiring infections from microorganisms. Various mechanisms that operate in the insect system to combat the diseases by producing antibacterial proteins and their gene regulation in producing various other proteins to counteract the invasion of pathogens have been documented in many insects and silkworms, but there is a paucity of literatures to show the mechanism of humoral responses to LPS in silkworms and especially in various breeds of *Bombyx mori* need to be studied to understand the biochemical factors by which the immune competence of them can be used as a dependable biochemical makers to estimate the degree of resistance and the percentage of survival of them . Hence, this article focuses on short listing the various studies pertaining to humoral reactions as part of biochemical mechanisms by which the various breeds of the silkworm of *B. mori* develop their required resistance to the pathogen for better survival and higher yield in the following biochemical approaches of proteins and enzymes as markers associated with genetic resistance for survival.

Proteins as markers associated with genetic resistance for survival.

Antibacterial molecules such as cecropin, which destroys the bacterial membrane by disturbing its structure (Boman, 1991, 1995) and lysozyme, which degrades the bacterial cell wall (Daffre *et al*., 1994) were among the first identified. Later other type of microbe –inducible proteins, *e.g*., nonself –recognition molecules such as hemolin (Bettencourt *et al*., 1997) and proteases (Dimopoulos *et al*., 1997) were identified. Many bacterially inducible proteins with as yet unknown functions have also been reported (Hughes *et al*., 1983; Kang *et al*., 1996). These findings indicate that the immune response induced by bacterial infection involves the production of both antibacterial molecules and proteins with functions not yet fully understood. Some proteins involved in detoxification may be inducible by microbial infection. Lipophorin, for instance helps detoxify lipopolysacchardies (LPSs) originating from bacteria in the hemolymph of the silkworm, *Bombyx mori* (Kato *et al*., 1994). The major functions of lysozyme are to destabilize the cell by depolymerising the cell wall and thus to make the cell wall more easily accessible for other antibacterial substances (ABS) and to enhance lysis of bacteria killed by the ABS. Single cecropin molecules aggregate and build clusters, which form pores in the membranes of their target bacterial destabilizing the cell (Okada and Natori, 1985; Steiner *et al*., 1988; Jaynes, 1989). Further, the moricin and cecropins are simultaneously induced upon bacterial infection, and they can effectively eliminate the wide variety of invading bacteria species (Hara and Yamakawa, 1995). Compared to the proteins from other organs of silkworm, the number of proteins in silkworm hemolymph is more (Kumar *et al*., 2011). Hence, a study focusing on the investigations of induced proteins in the hemolymph of silkworm races/breeds challenged with LPS would be of great use to make them as biochemical markers for associating the immunity of the different silkworm races of the *Bombyx mori*. The innate immunity in invertebrates (especially insects) consists of humoral and celluar components. The humoral components include antimicrobial peptides, lectins and melanin. Significant antimicrobial proteins like, Lebocin, Moricin (Hara and Yamakawa, 1995), Cecropins, Lysozyme like proteins (LLPs) have been reported from silkworm *Bombyx mori* ranging from 35 Kda were noticed in Kolar gold, CSR_2 and in Pure Mysore. The cecropins are peptides which have a broad spectrum of activity against gram +ve and gram –ve bacteria (Ganz, 2003, Qu *et al*., 1982; Gotz ad Bowman, 1985; Boman and Hultmar, 1987, Gandhe, 2007).

Antibacterial proteins are induced, mainly in the fat body and hemocytes upon bacterial infection.Insect antibacterial proteins are heat stable and have a broad antibacterial spectrum. Many antibacterial proteins have been isolated from various insects and at least four types (cecropin,

attacin, lebocin and moricin) have been identified in *B. mori*. Cecropin consists of about 40 amino acid residues and is heat stable. Three subtypes of cecropin (A, B and D) have been reported from *B. mori*. Cecropins are active mainly against Gram-negative bacteria. It was demonstrated that cecropin forms ion channels in bacterial membranes and, as a result, bacteria are killed. Attacin is a glycine rich and has a molecular mass of 20 kDa. Attacin acts against growing Gram-Negative bacteria and was shown to inhibit synthesis of the bacterial outer membrane.

Another novel antibacterial protein designated moricin was isolated from the hemolymph of *B. mori* and it showed antibacterial activity against *Staphylococcus aureus*11. This protein consists of 42 amino acid residues and is highly basic. It had no significant similarity with other antibacterial proteins. Moricin has antibacterial activity against several Gram-negative and positive bacteria, with a higher activity against Gram-positive bacteria than cecropin B and is inducible upon bacterial injection. These results suggest that the protein is responsible for antibacterial activity against Gram-positive bacteria in *B. mori*. The effects of the protein on bacterial liposomal membranes indicate the target of the protein is the bacterial cytoplasmic membrane. Two lectins have been reported from *B. mori*; one has a molecular mass of 260 kDa and another designated hemocytin 280 kDa (Jaynes, 1989).

In India, the indigenous tropical polyvoltine races showed more resistance to diseases than temperate bivoltine races (Moorthy *et al*., 2007). In order to understand the differential response at bio-molecular level, it may be an ideal approach to compare the expression level of antibacterial genes in hardy polyvoltine races like Pure Mysore and Nistari with temperate races, which are less hardy, and at the same time highly productive. Studies in this directions on bivoltine and polyvoltine silkworm breeds showed that the presence of induced proteins viz., Attacins, which ranges from 20-23 Kda as reported by Boman and Hultmar (1981) were noticed in silkworm breed *viz.*, CSR_2 (Suparna *et al*., 2011). Attacins has drastic effect on the permeability properties of the outer membrane of gram-ve bacterial (Carlsson *et al*., 1998) and the protein Lysozyme of 15-16 Kda sizes (Mohrig and Messner, 1968) were noticed in other silkworm breeds *viz.*, Kolar Gold and Pure Mysore. Hence, a study focusing on the investigations of induced proteins in the hemolymph of silkworm races/breeds challenged with LPS would be of great use to make them as biochemical markers for associating the immunity of the different silkworm races of the *Bombyx mori*.

Enzyme markers associated with genetic resistance for survival.

The melanin is synthesized by the activation of the pro phenoloxidase activating system (Soderhrall, 1982) comprising a complex cascade of serine proteases that allows the conversion of proPO to phenoloxide (PO), which, in turn, acts on substrates such as tyrosine and its derivatives (DOPA and dopamine) to form melanin. This process was extensively studied for the first time in crustacean *Astacus astacus* (Soderhrall, 1982) and in the insect *Bombyx mori* (Ashida and Ohnishi, 1967, Ashida *et al*., 1983). The proPO System has also been seen as a recognition system activated by different foreign materials, such as lipopolysaccharides and peptidoglycans from microbial cell walls (Söderhräll and Cerenius, 1998). Cuticular PO is normally considered to be injury PO, however, other two types of PO are present in the cuticle of insects: granular PO involved in the body colour and laccase-type PO involved in sclerotization of a newly ecdysed cuticle. Studies on inducible proteins revealed that some degrading enzymes, such as esterase may also help eliminate toxic molecules generated during microbial infection (Shiotsuki and Kato, 1999). It was also found that carboxylesterases are involved in the mechanism of insecticide resistance in insects (Oppenoorth, 1985).

The carboxyl esterase act as immune defense molecules against bacteria also degrade insecticides and continuous exposure to insecticides over several generations has resulted in the selection of carboxylesterase that specialize in insecticide degradation. Carboxyl esterase isozymes have been reported in mammal macrophages and monocytes, cells involved in the immune system (Munger *et al*., 1991; Scott *et al*., 1992). Studies on induction of carboxylesterase isozymes in *Bombyx mori* by *E. coli* infection showed an inducible proteins/isoforms of enzymes *viz*., Carboxyl esterase (CEs)–Est-1 and Est-2 – induced by the injection of *E. coli* or LPS. They were found with a similar other known bacterially inducible proteins and CEs clearly differed from noninducible CEs (Est-3, 4 and 5 which were visualized in the haemoloymps of silkworm in the native gel) in migration on analytical native PAGE and inhibitor sensitivity studies. These results suggest that Est-1 and Est-2 are novel CEs that are inducible either by bacterial or bacterial ligands (LPS) injections into the silkworms (Shiotsuki and Kato, 1999). Hence, a study focusing on investigations of induced isozymes in the hemolymph of silkworm races/breeds challenged with LPS would be of great help to make use of them as biochemical markers for associating the immunity of different silkworm races of the *Bombyx mori*.

CONCLUSION

The present article focuses on the use of biochemical markers that attribute to immuno competence in silkworms, which are qualitative and are reproducible. The biochemical markers indicative of immune competence of various silkworm breeds can be of use to identify genetically hardy breeds that can be better exploited in varied agro climatic conditions for raising disease free crop and thus silkworm improvement. In summary, the practical utility of the studies on protein and enzyme markers reveal that different type of proteins induced upon LPS treatment and different isoenzymes of carboxyl esterase and phenoxidases considered as key biomolecules which are of utmost importance to associate immune competence of the silkworm breeds as they are involved very much in either neutralizing or degrading the toxins generated by bacterial or LPS molecules in the cellular/humoral systems of the silkworms.

In view of the above various studies cited in the literatures in the various context of the experimental investigations in many insects in general and in silkworms in particular revealed that inducible proteins *viz*., cecrobin, lebonin *etc.*

and inducible isoforms of enzymes *viz*., carboxyl esterase and phenoloxidase under the effect of LPS in the system are the reliable and reproducible biochemical markers and can be used to rate the degree of resistance by their presence /absence through various recent advance biochemical techniques to diseases and thereby estimating genetic hardiness of individual breeds of the silkworm by which an immune competence that confer higher survival ability of the breeds may be used as dependable marker for screening large number of germplasm materials to list out genetically resistant breeds which are otherwise termed as genetically hardy. The understanding of the impact of bacterial ligand LPS as an immune elicitor on immune eliciting responses in silkworms and the role of key biomolecules on the mechanism of disease resistance revealed through the presence of an inducible proteins *viz*., cecropin, attacin, lebocin and moricin and isoforms of enzymes *viz*., carboxyl esterase and prophenoloxidase isoforms as effective reproducible markers would favor us in enhancing our theoretical level and their implications in understanding of the biochemical mechanism of immunity to guide the breeding of silkworm races which are stronger in immuno competence and as reflected from higher survival rate from a given agro climatic conditions is needed by any researchers / breeders to handle the germplasm material for achieving the ultimate goal of tapping the maximum genetic potentials of the silkworm genetic resources of the country.

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