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Entomology as a Science and IPM as a Technology - the Way Forward

November 14-15, 2014

Compilation of Invited Lectures

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held at

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Disrupting Insect Luminal Enzymes for Pest Management

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ABSTRACT

Selective inhibition of digestive enzymes in insects forms an alternative approach in innovative pest management. Proteins are usually digested in the insect gut by enzymes that are active in fairly alkaline (Lepidoptera) to slightly acidic *p*H (Coleoptera). Serine proteases particularly trypsin and chymotrypsin play major roles in effective digestion in a wide array of insects. Plant proteinase inhibitor proteins elicited in response to insect attack affect the digestibility of ingested proteins, decreasing the amounts of free amino acids required for growth, development and reproduction. Such plant-derived proteinase inhibitors (PI) are part of the plant's natural defense system against insect predation. Transgenic plants expressing PI is an useful adjunct to the use of Bt as biopesticide. Case studies of luminal proteases in cardamom shoot and capsule borer and coconut red palm weevil and their inhibition using PI is discussed. Exploitation of trypsin modulating oostatic factor in crop pests as revealed in mosquitoes is emphasized. Development of new types of PI by smart engineering of proteins with altered affinity for different proteases and incorporating multi domain PIs in a single gene would be used for combating adaptive responses in insect.

INTRODUCTION

Extensive and exclusive use of chemical pesticides especially at very high concentration may result in rapid build-up of resistance to such compounds, but their non-selectivity affects the balance between pests and natural predators and generally favours the pests (Metcalf, 1986). Synthetic insecticides not only increase production costs but also cause environmental hazards. Therefore, heavy reliance on this single strategy is highly unsafe,

and the timely development of alternative complementary methods to chemical control is advisable. Development of an environmental-friendly control method is, therefore, a major goal of researchers in pest management. Currently some of the major aspects in pest control are to achieve the selective inhibition of the digestive enzymes of many insect pests.

LUMINAL ENZYMES

It is well established that proteolytic enzymes in insect guts are primarily responsible for the digestion of plant proteins. Since insects are unable to synthesize a number of amino acids, they depend on digestive proteinases and plant proteins to meet their nutritional requirements (Bernays and Woodhead, 1984). Proteins are digested in the insect gut by enzymes that are active in fairly alkaline (Lepidoptera) to slightly acidic *p*H (Coleoptera) (Applebaum, 1985). Protein breakdown in insect guts is mediated by the concerted action of digestive proteases, particularly trypsin-like (peptidase activity) and chymotrypsin-like proteases. Serine proteases, including trypsin and chymotrypsin, are known to be the most important digestive enzymes in the larval gut and account for about 95% of digestive activity (Sreenivasan *et al.,* 2006).

Trypsin and elastase-like chymotrypsin were the predominant digestive proteinases of cardamom shoot and capsule borer, *Conogethes punctiferalis* and age related modulation of midgut proteinases existed for trypsin, chymotrypsin, elastase-like chymotrypsin and leucine aminopeptidase (Josephrajkumar *et al.*, 2006). Lepidopteran larvae have been extensively studied because of their overwhelming impact as pests on economically important plants. Proteinases are divided into four classes based on the amino acid or metal ion involved in the catalytic site which cleaves peptide bonds; serine, cysteine, aspartic and metalloproteases. All these classes of proteases have been demonstrated in insects (Terra and Ferreira, 1994). Serine proteases have been identified from the digestive tracts of insects from many families and many of these enzymes are inhibited by protease inhibitors. Additionally, serine protease inhibitors have an anti-nutritional effect on several insect species (Ussuf *et al.*, 2001).

Being essentially indispensable to the maintenance and survival of their host organism, proteases play key roles in many biological processes and can be potentially damaging when over-expressed or present in higher concentrations, and their activities need to be correctly regulated (Habib and Fazili, 2006). For this reason the activities of these enzymes need to be strictly

regulated and controlled. Proteases being major regulatory enzymes play a prominent house keeping role in the cell physiology of all living systems.

PROTEINASE INHIBITORS

Plant proteinase inhibitor proteins elicited in response to insect attack affect the digestibility of ingested proteins, decreasing the amounts of free amino acids required for growth, development and reproduction (Barrett, 1986). As a defense mechanism, host plants produce proteinase inhibitors (PI) to suppress the activity of serine proteinases. Plant-derived proteinase inhibitors are of a particular interest because they are part of the plant natural defense system against insect predation. Previous studies on the effect of dietary proteinase inhibitor either artificially introduced into defined diets or already present in plant tissues, have shown that these PI can be detrimental to growth and development of a wide range of insects (Ryan, 1989; Hilder *et al.*, 1987).

The first PI gene to be successfully transferred to tobacco, resulting in enhanced resistance against *Manduca sexta* was cowpea trypsin inhibitor (CpTI) which provided direct evidence for the effectiveness of CpTI against a pest. The efficiency of transgenic tobacco plants expressing CpTI was also tested effective against *Spodoptera litura*. Reduction to the extent of 50% was observed in the biomass of *S. litura* larvae fed on transgenic leaves expressing 3-5 mg CpTI/g fresh leaves (Hilder *et al.*, 1987). The most effective proteinase inhibitor in terms of retardation of growth of insects was aprotinin, bovine pancreatic trypsin inhibitor, which inhibits serine proteases such as trypsin, chymotrypsin, plasmin and kallikrein (Zhong *et al.*, 1999). Aprotinin inhibited the growth of Argentine stem weevil, *Listronotus bonariensis* when administered continuously in artificial diets to neonate or one-week-old larvae (Todd *et al.*, 2002). Inclusion of aprotinin (150 μM) in cardamom shoots used as feed reduced the body weight of *C. punctiferalis* larvae by day 4 (Josephrajkumar *et al.*, 2005).

The use of genetic engineering to produce pest resistant transgenic plants represents one of the many current approaches aimed at increasing agricultural productivity. Protease inhibitors are one group of candidate proteins being targeted for expression in cultivated plants for plant resistance. Protease inhibitors form stoichiometric complexes with specific proteolytic enzymes, thus preventing their catalytic function (Laskowshi and Kato, 1980). Since protease inhibitors are primary gene products, they are excellent candidates for engineering pest resistance into plants. Moreover, protease inhibitors have been used because of their small size, abundance, specificity and stability.

When expressed in plants, protease inhibitors can bind with key digestive proteases of insects feeding on plants, disrupting their digestion and reducing growth and survival (Gatehouse *et al.*, 2000).

Since proteolysis is an essential part of food digestion in insects, studies on insect proteases are important. Digestive proteinases of insects also catalyze the release of free amino acids from dietary protein and thereby provide a supply of nutrients essentials for normal growth and development. Disruption of protein digestion by protease inhibitors represents an alternative approach to pest management in a world dominated by chemical pesticides. This approach requires a thorough understanding of the biochemical properties of the proteases from the gut homogenate, characterization of these proteases particularly trypsin-like protease in relation to developmental stages and understanding the way it reacts with classical protease inhibitors such as soybean trypsin inhibitor and aprotinin.

CASE STUDIES

Cardamom shoot and capsule borer, *Conogethes punctiferalis* Guenée (Pyralidae : Lepidoptera)

C. punctiferalis is one of the key pests of cardamom causing damage to growing tillers as well as fruit capsules affecting the yield significantly. Individual guts were dissected out, homogenized and clarified for use in activity assays. Activities of serine-type endopeptidases, particularly trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), elastase-like chymotrypsin and one exopeptidase, leucine aminopeptidase (LAP) were assayed using the substrates, benzoyl-arg-*p*-nitroanilide (BA*p*NA), benzoyl-tyr-*p*-nitroanilide (BT*p*NA), succinyl-ala-ala-pro-leu-*p*-nitroanilide (SAAPL*p*NA) and leu-*p*-nitroanilide (L*p*NA), respectively. Protease inhibitors *viz.*, aprotinin, 0–1.67 nM soybean trypsin (Kunitz) inhibitor (SBTI) and 0.04–1.00mM phenylmethanesulfonyl fluoride (PMSF) were also evaluated *in vitro.*

Trypsin and elastase-like chymotrypsin were prominent digestive proteases, wherein the specific activities at early fifth-instar exceeded 320 nmole of *p*NA released $min^{-1}mg^{-1}$protein. BA*p*NA-ase (trypsin) activity was highest in early fifth-instar and lowest in late fifth-instar showing a 10-fold decrease in the specific activity as the insect advanced towards pupation. A similar trend was also observed for chymotrypsin, elastase-like chymotrypsin and leucine amino peptidase activities. Aprotinin at 3.33 nM showed 7-fold and 8-fold decrease in specific activities for early and mid stages of the *C. punctiferalis*,

respectively. Specific activities were reduced by 11- and 10-fold, respectively for the same stages with SBTI (1.67 nM). However, phenylmethanesulfonyl fluoride reduced the activities by only 3 and 2-fold, respectively at a higher concentration of 1mM for the same stages. It is therefore suggested that nanomolar concentrations of soybean trypsin inhibitor and aprotinin as well as phenylmethanesulfonyl fluoride at millimolar concentrations are needed to inhibit trypsin. Trypsin and elastase-like chymotrypsin were drastically and significantly inhibited by 94% and 29%, respectively by aprotinin (150 nM) under *in vitro* conditions. The chymotrypsin activity was too low to demonstrate meaningful inhibition by aprotinin.

Borer infested cardamom capsules

Boring caterpillar

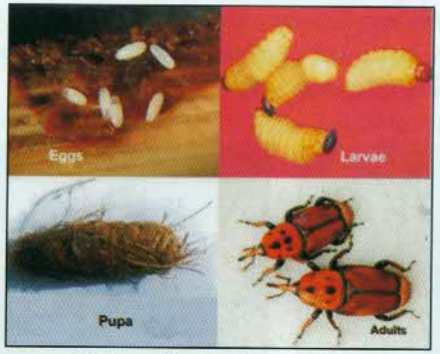
Adult moth

Together, these results suggest that, in addition to proteinase inhibitor specificity, the developmental stage of *C. punctiferalis* and the concentration of the inhibitor applied may also be important factors in determining the efficacy of the inhibitor against trypsin and elastase activities. A significant amount of inhibitors may have to be ingested during the early and active feeding stages of the test insect coinciding that with highest levels of activity of digestive proteases. This is because of reduced activities of midgut proteases when the insect approached pupation. As the larvae approached pupation, lower levels of proteolytic activity are present in the insect guts, concomitant with decreased feeding activity (Josephrajkumar *et al.*, 2006).

Coconut Red palm weevil, *Rhynchophorus ferrugineus* (Olivier) [Curculionidae: Coleoptera]

R. ferrugineus, a concealed tissue borer, is a lethal pest of palms and is reported to attack 17 palm species world wide. Currently, the pest is reported in 15% of the coconut-growing countries and in nearly 50% of the date palm-growing countries. Infested palms, if not detected early and treated, often die. Three different stages of the test insect (early, mid and late-instar) coinciding

the physiological stages of development and appropriate age were selected for extraction of gut. Peptidase and elastase-like chymotrypsin activities were assayed using BA*p*NA (1 mM) and SAAPL*p*NA (1 mM) as substrates, respectively. Inhibition assays were carried out using aprotinin (0-50 µg), and soybean trypsin inhibitors (0- 50µg) and phenyl methyl sulphonylfluoride (0-1700 µg), which are the classical inhibitors of serine protease.



Biology of red palm weevil

Crown toppled palm

Investigations on luminal proteinases of grubs of red palm weevil, *R. ferrugineus* infesting coconut revealed the presence of two endodpeptidases *viz*., trypsin (BA*p*NA-ase activity) and elastase-like chymotrypsin (SAAPL*p*NA-ase activity) in all stages of larval development. Highest activity of these peptidases coincided with the active feeding stage (mid-larval stage) of the insect. Results indicated that 50 µg of aprotinin, 50 µg of Soybean Trypsin Inhibitor (SBTI) and 1700 µgof Phenyl Methyl Sulphonyl Fluoride (PMSF) induced inhibitory effect to the tune of 77.4%, 63.1% and 55.9% respectively, on peptidase activity of *R. ferrugineus*. Aprotinin was found to be more inhibitory than SBTI and PMSF evinced least inhibition for the given concentration of inhibitor tested on the peptidase activity of *R. ferrugineus*. Serine proteinase inhibitors *viz*., aprotinin (50 µg), SBTI (50 µg) and PMSF (1700 µg) had a marginal reduction 32%, 14% and 11% respectively on elastase-like chymotrypsin activity of *R. ferrugineus* suggesting the serine nature of the protease. Among the inhibitors evaluated, inhibition pattern of elastase activity on the crude mid gut homogenate of *R. ferrugineus* was found to be aprotinin > soybean trypsin inhibitor > phenyl methyl suphonyl fluoride in that order of magnitude. *In vivo* bioassay of 250 µM aprotinin on coconut petiole method using early stage grubs of *R. ferrugineus* indicated a significant weight loss of 18.9% due to incorporation of serine protease inhibitor, aprotinin in a period of 120 h (Josephrajkumar *et al.,* 2013).

Lepidopteran gut (*Opisina arenosella*)

Coleopteran gut (*R. ferrugineus*)

TRYPSIN MODULATING OOSTATIC FACTOR

Borovsky and coworkers identified and characterized the peptide hormones that down regulates expression of trypsin-like serine proteases which are primary insect digestive proteases. These peptides form the core technology upon which Insect Biotechnology Inc., California is developing a variety of products to address significant market segments.

Mosquitoes such as yellow fever mosquitoe, *Aedes aegypti* require the digestion of blood meal for egg development. The blood meal triggers the biosynthesis of midgut serine proteases which are responsible for digestion of the blood. Free amino acids liberated in the intestine are transported to the fat body for the biosynthesis of vitellogenin which in turn is transported to the oocytes for their development. After 18-24h, after the start of the blood meal the follicular epithelial cells in the ovary begin to synthesize a decapeptide, called Trypsin Modulating Oostatic Factor, (TMOF) (Borovsky *et al*., 1993). TMOF is released into the haemolymph where it binds to specific receptors on the epithelial cells of the midgut, and thereafter the trypsin biosynthesis ceases, no blood is further digested, no more amino acids are released, no vitellogenin is synthesized and egg development is arrested. Northern Analysis revealed that injection of the endogenous peptide in this species does not inhibit the transcription of the mRNA of trypsin but its translation *ie*., the biosynthesis of trypsin in inhibited (Borovsky *et al*., 1996).

When TMOF is administered to adult mosquitoes *via* their diet, it is apparently not activated in the gut. It can pass the gut epithelial cells reach

the haemolymph bind to the receptors located on the membrane of the midgut epithelial cells, stop biosynthesis of trypsin and terminate egg development. When TMOF is absorbed on yeast particles and fed to the larvae, a high dosage (1 ng per larva) is needed for the lethal effect in the laboratory. TMOF gene can be fused into the coat protein gene of Tobacco Mosaic Virus together with an appropriate cleavage site for trypsin. Only 140 pg per larva of this construct is necessary to inhibit proteases and the larvae die within 5 days (Borovsky *et al.*, 1996). Another genetic engineering project used the alga *Chlorella* into which the TMOF gene had been incorporated. After feeding this alga to mosquito larvae, trypsin biosynthesis stops and death occurs within 72h.

PRACTICAL UTILITY IN PEST MANAGEMENT

(i) Development of PI as botanical biopesticide

(ii) Bio-engineering effective PI and expression in plants against borer attack

(iii) Gene pyramiding with Bt

(iv) Application in RNA interference tool

(v) Exploitation of TMOF in crop pests

(vi) Smart engineering and incorporating multi domain PIs in a single gene

ADAPTIVE RESPONSES

Adaptive response by insects to ingested protease inhibitors may complicate inhibitor based control strategies. A typical insect midgut contains an estimated 1020 different proteases (Bown *et al.*, 1997), which are differentially regulated and all cannot be inhibited by protease inhibitors. Insects also appear to adapt to protease inhibitors by compensatory mechanisms (Oppert *et al.,* 2002). These include an increased production of inhibitor-sensitive proteases or synthesis of novel inhibitor-insensitive proteases. Successful development of protease inhibitors that can be used as pest control agents requires knowledge of the inhibitory pattern of these inhibitors against each class of proteases within the family of insect species (Zhu and Baker, 1999). Some coleopteran larvae have demonstrated resistance to protease inhibitors by resorting to inhibitor proteolysis (Girard *et al.*, 1998). In this context, every protease inhibitor should be bio-assayed for the effect on larval growth and survival before developing strategies for insect pest management using protease inhibitors (Oppert *et al.*, 2002). In order to employ an effective pest control strategy, it is very important to achieve multiple inhibitor expressions in a concerted manner.

While pest-resistant transgenic plant cultivars currently available commercially employ only Bt toxin genes, the development of transgenic plants expressing protease inhibitors has emerged in recent years as an additional strategy for pest management (Hilder *et al.*, 1987). Protease inhibitor derived genes are found to have the advantage of efficient expression in transgenic plants (Ussuf *et al.*, 2001). Although the exact mode of action of protease inhibitors is complex, it is fundamentally different from that by which Bt toxins operate. Transgenic plants expressing protease inhibitors may therefore be a useful alternative or adjunct to the use of Bt as a biopesticide. Thus, it seems likely that a transgenic plant expressing aprotinin or an equivalent protease inhibitor could be protected from attack by a susceptible pest species.

CONCLUSION

The technology for developing insect-resistant transgenic plants is expanding very widely. Such plants will become a part of IPM systems in future. Since codon usage of Bt is sub-optimal for expression in plants, it therefore calls for synthetic genes suitable for plant expression. Plant derived genes such as PI would become handy for efficient expression in transgenic plants. To counter Bt resistance, it was suggested to utilize genes encoding PIs in combination with Bt. Results of gene pyramiding strategies are quite encouraging. Development of new types of PI by smart engineering of proteins with altered affinity for different proteases and incorporating multi domain PIs in a single gene are attractive strategies for exploiting gut proteinases in pest management.

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