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STRUCTURAL AND FUNCTIONAL DIVERSITIES IN LEPIDOPTERAN SERINE PROTEASES [#]

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Abstract: Primary protein-digestion in Lepidopteran larvae relies on serine proteases like trypsin and chymotrypsin. Efforts toward the classification and characterization of digestive proteases have unraveled a considerable diversity in the specificity and mechanistic classes of gut proteases. Though the evolutionary significance of mutations that lead to structural diversity in serine proteases has been well characterized, detailing the resultant functional diversity has continually posed a challenge to researchers. Functional diversity can be correlated to the adaptation of insects to various host-plants as well as to exposure of insects to naturally occurring antagonistic biomolecules such as plant-derived protease inhibitors (PIs) and lectins. Current research is focused on deciphering the changes in protease specificities and activities arising from altered amino acids at the active site, specificity-determining pockets and other regions, which influence activity. Some insight has been gained through *in silico* modeling and simulation experiments, aided by the limited availability of characterized proteases. We examine the structurally and functionally diverse Lepidopteran serine proteases, and assess their influence on larval digestive processes and on overall insect physiology.

Key words: Functional diversity, Lepidoptera, Serine protease, Structural diversity

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Invited paper

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Abbreviations used: AI – amylase inhibitor; *Bt – Bacillus thuringiensis;* HGPs – *Helicoverpa armigera* gut proteases; PI – protease inhibitor.

INTRODUCTION

The ubiquitous nature of Lepidopteran herbivores contributes significantly to insect pest-mediated crop damage in a wide variety of agronomically important plants (Tab. 1). The extensive use of pesticides has led to a selection of resistant insect species, which have already diversified into single host-plant specific pests (monophagous) and multiple host-plant pests (polyphagous). The larvae of herbivorous insects feed actively on plants to gather nutritional components required for development and progression into the reproductive adult phase. Herbivorous Lepidopteran larvae feed voraciously on plant parts to derive nutrients for optimum growth and development. The primary constituent of their diet is protein, which is digested into amino acids by proteases. Similarly, complex polysaccharides are broken down into simple sugars by amylases. The monomeric forms, i.e., amino acids and sugars, are absorbed and assimilated for growth processes that lead to normal development into healthy adults. Any impairment to digestion by antagonistic agents like proteinase inhibitors (PIs) and amylase inhibitors (AIs) leads to developmental malformations. Other agents, like lectins, which affect nutrient absorption across the midgut epithelium, also yield similar effects. Hence, most insect control programs focus on the larval phase as a target. Among these strategies, PI-based approaches usually focus on the dominant mechanistic class of digestive protease in Lepidoptera, i.e., serine proteases. Although much has been understood about these proteases, the overwhelming complexities in their structure and function leave much to be explored. We discuss some of the implications of this diversity.

MULTIPLE PROTEASE SPECIFICITIES AND ISOFORMS IN LEPIDOPTERA

The Lepidopteran larval midgut hosts a complex proteolytic environment of various specificities, with, among others, trypsins, chymotrypsins, elastases, cathepsin-B like proteases, aminopeptidases and carboxypeptidases, which are all responsible for protein digestion. Serine proteases are known to dominate the larval gut environment and contribute to about 95% of the total digestive activity. Beneath the complexity of multiple protease specificities, there usually exists an array of diverse protease isoforms; for example, the gut of *Helicoverpa armigera* alone is known to contain about twenty different types of active serine protease isoforms at any given moment [1-3]. This multitude of isoforms does seem unnecessary, especially when only a few of them (e.g., trypsins) contribute significantly to digestion. However, a broader array of proteases with (almost) similar specificities could be advantageous to the insect in dealing with the diverse plant protein content, which may be recalcitrant or even toxic, given a narrow choice of proteases. It is interesting to note that the feeding pattern of larvae [4] and the complement of gut proteases do not remain constant during

Tab. 1. Major Lepidopteran insect pests, their target crops and their primary digestive proteinases

Insect Pest		Target Crop(s)	Digestive proteases*
Scientific name(s)	Common name(s)		
Helicoverpa armigera, Helicoverpa zea, Heliothis virescens	Podborer, Tobacco budworm, Corn earworm, Tomato fruitworm, Sorghum headworm, Cotton bollworm	Among the 180 different reported hosts, the major ones are: Alfalfa (*Medicago sativa*), Broccoli (*Brassica oleracea* var. *italica*), Cabbage (*Brassica oleracea*), Chickpea (*Cicer arietinum*), Chrysanthemum (*Chrysanthemum coronarium*), Cotton (*Gossypium hirsutum*), Gardenpea (*Pisum sativum*), Kidney beans (*Phaseolus vulgaris*), Lentils (*Lens culinaris*), Lettuce (*Lactuca sativa*), Maize (*Zea mays*), Okra (*Abelmoschus esculentus*), Peanut (*Arachis hypogea*), Pepper (*Capsicum annum*), Potato (*Solanum tuberosum*), Pigeonpea (*Cajanus cajan*), Sorghum (*Sorghum bicolor*), Soybean (*Glycine max*), Strawberry (*Fragaria virginiana*), Sweet potato (*Ipomoea batatas*), Tobacco (*Nicotiana tobacum*), Tomato (*Lycopersicon esculentum*), Watermelon (*Citrullus lanatus*)	trypsin (90%), chymotrypsin (5%), elastase (1%), carboxypeptidase (1%), aminopeptidase (1%), cathepsin B-like (1%), metalloprotease (1%)
Spodoptera litura Spodoptera exigua *Spodoptera frugiperda*	Tobacco cutworm, Cotton bollworm, Beet armyworm, Fall armyworm	Beet (*Beta vulgaris*), Cabbage, Cotton, Cowpea (*Vigna unguiculata*), Eggplant (*Solanum melongena*), Gardenpea, Kidney bean, Onion (*Allium cepa*), Peanut, Pepper, Potato, Radish (*Raphanus sativus*), Safflower (*carthamus tinctorius*), Soybean, Sweet potato, Tobacco, Tomato	trypsin (7%), chymotrypsin (85%), elastase (1%), aminopeptidase (5%) carboxypeptidase (1%)
Manduca sexta	Tobacco hornworm	Eggplant, Tomato, Tobacco	trypsin (10%), chymotrypsin (80%), elastase (1%), aminopeptidase
Pectinophora gossypiella	Pink bollworm	Cotton	(none reported)
Pieris rapae	Imported cabbageworm	Alfalfa (*Medicago sativa*), Broccoli, Brussels sprout (*B. oleracea* var. *gemmifera*), Cabbage, Cauliflower (*B. oleracea* var. *botrytis*), Horseradish (*Armorecea rusticana*)	(none reported)
Euxoa auxiliaries	Army cutworm	Alfalfa, Barley (*Hordeum vulgare*), Cabbage, Flax (*Linum usitatissimum*), Mustard (*Brassica nigra*), Peas, Wheat (*Triticum aestivum*),	(none reported)
Plutella xylostella	Diamondback moth	Broccoli, Brussels sprout, Cabbage, Cauliflower, Horseradish, Mustard (*Brassica nigra*)	trypsin (major), chymotrypsin (major), elastase, aminopeptidase
Agrotis ipsilon	Black cutworm	Broccoli, Cabbage, Carrot (*Daucus carota*), Eggplant, Green beans, Mustard, Potato, Spinach (*Spinacea oleracea*), Sugarcane (*Saccharum officinarum*)	trypsin (major), chymotrypsin
Anticarsia gemmatalis	Velvetbean caterpillar	Cowpea, Horsebean (*Parkinsonia aculeate*), Peanut, Soybean, Velvet bean (*Mucuna pruriens*),	trypsin (major), chymotrypsin, cathepsin B-like

* Figures in parentheses represent approximate percentage contribution to total gut protease activity.

larval growth [3]; on legumes like chickpea (*Cicer arietinum*), lower instar larvae feed more on the leaf and flower tissue, whereas the higher instar larvae feed on developing seeds, which have a quantitatively higher and qualitatively different protein content. Owing to changes in feeding preferences as well as the tissue-specific variation of plant proteins, a dynamic modulation of larval digestive proteases seems sensible for achieving the optimal digestion of dietary protein that is required for normal growth and development. Thus, developmentally regulated proteinase gene expression would seem important for larval survival. Indeed, it has been experimentally verified that the regulation of certain key proteases in *H. armigera* larvae is independent of dietary composition, and follows growth and development [5]. Some Lepidopterans exhibit an amazing flexibility in adapting to various host plants (polyphagy) by altering the specificities of their gut proteases in response to qualitative changes in dietary protein content and when the existing proteases are ineffective and/or inefficient for digestion [3]. Studies on insect responses to the dietary incorporation of plant-derived proteinase inhibitors (PIs) have indicated a biphasic response characterized by an initial upregulation of all digestive protease specificities, which precedes a simultaneous downregulation of PI-sensitive proteases and upregulation of PI-insensitive proteases [6]. A similar response can be expected with a change in host plant. Thus, the significance of the differential expression of digestive proteases can never be underestimated. However, in spite of being a commonly observed phenomenon, the exact nature of the signaling mechanism that governs the differential regulation of protease genes is not well understood. The existence of 'monitor-peptides', and their putative contribution to the differential expression of gut proteases remains unproven [6], and thus the molecular basis of larval responses remain enigmatic. However, in Lepidoptera, certain neuropeptides have been identified that appear to function like vertebrate pancreatic peptides [7-9]; they possess the ability to "flick the switch" that governs digestive protease expression. Although a direct correlation might not be possible between digestion in insects and higher animals, useful insight can definitely be gained from such parallel analyses, especially in deriving models to explain digestive processes. Studies on insect responses aimed at identifying messenger molecules are well justified and would eventually aid in understanding the complex signaling events that are responsible for monitoring and coordinating nutrient uptake and gut proteolytic activities.

MOLECULAR DIVERSITY VERSUS FUNCTIONAL DIVERSITY

Information on the primary structure of polypeptides, i.e. the sequence of amino acids, usually formed the preliminary basis for further studies that revealed a considerable structural diversity in proteases, attributable to natural mutation events and selection of functionally active variants. The presence of multiple protease isoforms can be traced back to multi-copy protease genes that probably

arose in the insect genome due to gene duplication and diversification events [10]. It has been proposed [11] that the higher success rate in the incorporation of serine residues into catalytic centers coupled with the independent evolution of the various serine protease 'clans' are responsible for the higher diversity observed in serine proteases. Attempts have also been made to associate structural motifs as 'markers' to trace the evolutionary history of serine proteases and the inter-relationships between their various specificities [12]. The impact of evolution on diversity in serine proteases appears to be positive in that this mechanistic class forms the dominant population in the Lepidopteran digestive environment. It is common knowledge that mutation events that lead to amino acid alterations influence the structural and functional properties of the translated polypeptides. Although it is possible to visualize the mutations by determining the changes in amino acid composition and to explain the presence of structural isoforms, this does not necessarily provide any information on altered activities. This has been routinely observed in studies pertaining to the structural diversity of insect gut proteases in model insects [10, 13] where the determination of the biologically more relevant functional diversity resulting from these changes has always faced numerous obstacles. Though newly identified putative proteases are routinely annotated based on sequence similarity to known proteases, this may not always be accurate, and at worst may be totally misleading when it comes to predicting their function. In fact, experimentally derived data on substrate specificity could eventually narrate other facts. Thus, anomalies such as 'functional variants' (proteases homologous to one particular type but having activity similar to another) are routinely observed, as exemplified in Fig. 1, which shows a few representative Lepidopteran serine proteases. It is seen in case of an elastase from *Manduca sexta* (mse_ela_1, AAA67842), which is similar to a chymotrypsin from *Heliothis virescens* (hvi_chy_1, AAF43709). Another example would be the chymotrypsin from *H. armigera* (har_chy_8, CAA72951), which seems to be unrelated to other chymotrypsins. Newly identified putative serine protease gene sequences are at risk of being mis-annotated until their products are functionally characterized. Hence, the activity-characterization of the other serine proteases in our example, i.e. those from *Bombyx mori* (bmo_ser_1, BAD93199; bmo_ser_5, BAB91156), *H. armigera* (har_div_2, CAA72965; har_ser_2, AAD31713) and *Lonomia oblique* (lob_ser_1, AAV911432; lob_ser_3, AAV91434; lob_ser_4 AAV91435; lob_ser_5, AV91456; lob_ser_6, AAV91457; lob_ser_7, AAV91544), which form a separate structural group, could further elucidate this feature. Although direct experimental evidence is always preferred for functional characterization, these approaches involve intricate procedures that are often laborious and time consuming. In the absence of a credible means to correlate structural and functional aspects, a parallel trend of theoretical studies has also gained momentum; approaches include study of structural characteristics by modeling [14], analysis of criteria that govern the geometry and function of the active site [15], and parallel dissection of structural

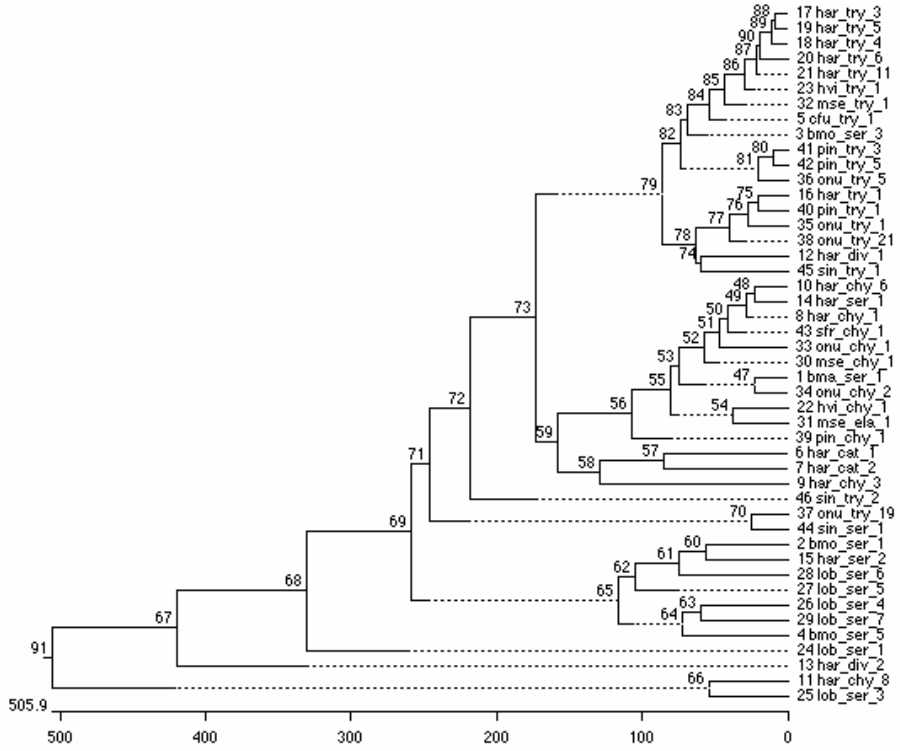

Fig. 1. Do sequence variations reflect functional diversity? Non-redundant Lepidopteran endopeptidase sequences were aligned using the Clustal algorithm and an unrooted phylogenetic tree was derived based on sequence similarities. Bootstrap values are indicated. Known activities were compared to determine whether they co-relate to homologous sequences. *Bombyx mandarina* serine protease (bma_ser_1, AAX39408); *B. mori* serine proteases (bmo_ser_1, BAD93199; bmo_ser_3, AAB26023; bmo_ser_5, BAB91156); *Choristoneura furmiferana* trypsin (cfu_try_1, AAA81525); *Helicoverpa armigera* cathepsins (har_cat_1, AAQ75437; har_cat_2, AAF35867); *H. armigera* chymotrypsins (har_chy_1, CAA72960; har_chy_3, CAA72966; har_chy_6, CAA72958; har_chy_8, CAA72951); *H. armigera* serine proteases (har_div_1, CAA72953; har_div_2, CAA72965; har_ser_1, AAC02217; har_ser_2, AAD31713); *H. armigera* trypsins (har_try_1, AAR20817; har_try_3, CAA72956; har_try_4, CAA72955; har_try_5, CAA72954; har_try_6, CAA72949; har_try_11, CAA72957); *Heliothis virescens* chymotrypsin (hvi_chy_1, AAF43709); *H. virescens* trypsin (hvi_try_1, AAF43708); *Lonomia oblique* serine proteases (lob_ser_1, AAV91432; lob_ser_3, AAV91434; lob_ser_4, AAV91435; lob_ser_5, AAV91456; lob_ser_6, AAV91457; lob_ser_7, AAV91544); *Manduca sexta* chymotrypsin (mse_chy_1, AAA58743); *M. sexta* elastase (mse_ela_1, AAA67842); *M. sexta* trypsin (mse_try_1, P35047); *Ostrinia nubilalis* chymotrypsins (onu_chy_1, AAX62040; onu_chy_2, AAX62031); *O. nubilalis* trypsins (onu_try_1, AAX63384; onu_try_5, AAX62036; onu_try_19, AAR98921; onu_try_21, AAR98919); *Plodia interpunctella* chymotrypsin (pin_chy_1, AAC36149); *P. interpunctella* trypsins (pin_try_1, AAF24228; pin_try_3, AAF24226; pin_try_5, AAC36248); *Spodoptera frugiperda* chymotrypsin (sfr_chy_1, AAO75039); *Scirpophaga incertulas* serine protease (sin_ser_1, AAC02219); *S. incertulas* trypsins (sin_try_1, AAC02220; sin_try_2, AAC02218).

characteristics and activities in related proteases [16]. Likewise, *in silico* simulation experiments on protease activity [17] and protease-protease inhibitor interactions [18, 19] have also provided useful insights. These studies are based on experimental evidence derived from known sequences and specificities of purified proteases. The activity of serine proteases is a function of the "catalytic triad" which results from the spatial proximity of histidine (57), aspartate (102) and serine (195) residues in the polypeptide. This triad is a well-conserved feature of serine proteases and its mechanism has been completely elucidated [20-22]. Studies on the role of individual amino acids (that form the catalytic triad) by site-directed mutagenesis as well as in atypical serine proteases [23-25] have contributed greatly to our current level of understanding. The specificity of the serine proteases is, however, not governed by the catalytic triad, but is rather due to a molecular (S1) pocket that interacts with the side chains of the amino acids that lie in the cleavage (P1-P1') site of the substrate; as an extrapolation, the amino acids in the S1 pocket may be linked to functional diversity, as they identify and interact with various substrate polypeptides. In the case of trypsins, the aspartate (189), glycine (216) and glycine (226) residues contribute to a negatively charged S1 site, so arginine or lysine is preferred at P1 on the substrate. Similarly, serine (189), glycine (216) and glycine (226) form a deep hydrophobic pocket in chymotrypsins leading to a preference for phenylalanine at P1. In addition, adventitious contacts resulting from amino acids at sites other than the S1 pocket influence the kinetics of substrate binding and inhibition [26] – in fact, sub-sites of the trypsin catalytic triad that are known to influence substrate hydrolysis by selectively binding to the substrate or intermediate form(s) [27] can interact with up to position P_{12} of the substrate [28]. In Fig. 2A and Fig. 2B, where we laid out the amino acid variations across representative Lepidopteran trypsins and chymotrypsins, key changes have been illustrated. It is clearly seen that the amino acids that form the catalytic triad (marked as '●') are highly conserved in trypsins and chymotrypsins, as are those of the oxyanion hole (marked as '⇒'); an exception is the trypsin from *Ostrinia nubilalis* (onu_try_6), where aspartate (194) has been replaced by a glycine. This feature reiterates the importance of the conserved amino acids in the catalytic triad. The amino acids are characteristically well conserved at the S1 pocket (grey) in trypsins, unlike in the chymotrypsins, which show a much greater degree of diversity. We would thus expect chymotrypsins to exhibit greater flexibility in substrate recognition and perhaps in activity as compared to trypsins, though there is no data to currently support this hypothesis. This feature may be linked to the relative populations of serine protease isoforms in Lepidopterans like *H. armigera*, where the sheer diversity of trypsins probably compensates for their limited flexibility at the S1 pocket, whereas the higher flexibility expected in chymotrypsins could compensate for the relatively lower content. Although this hypothetical case has not been validated, it does provoke an interesting chain of thought for future investigations. The amino acids in the regions marked by horizontal braced parentheses are thought to be involved in the formation of

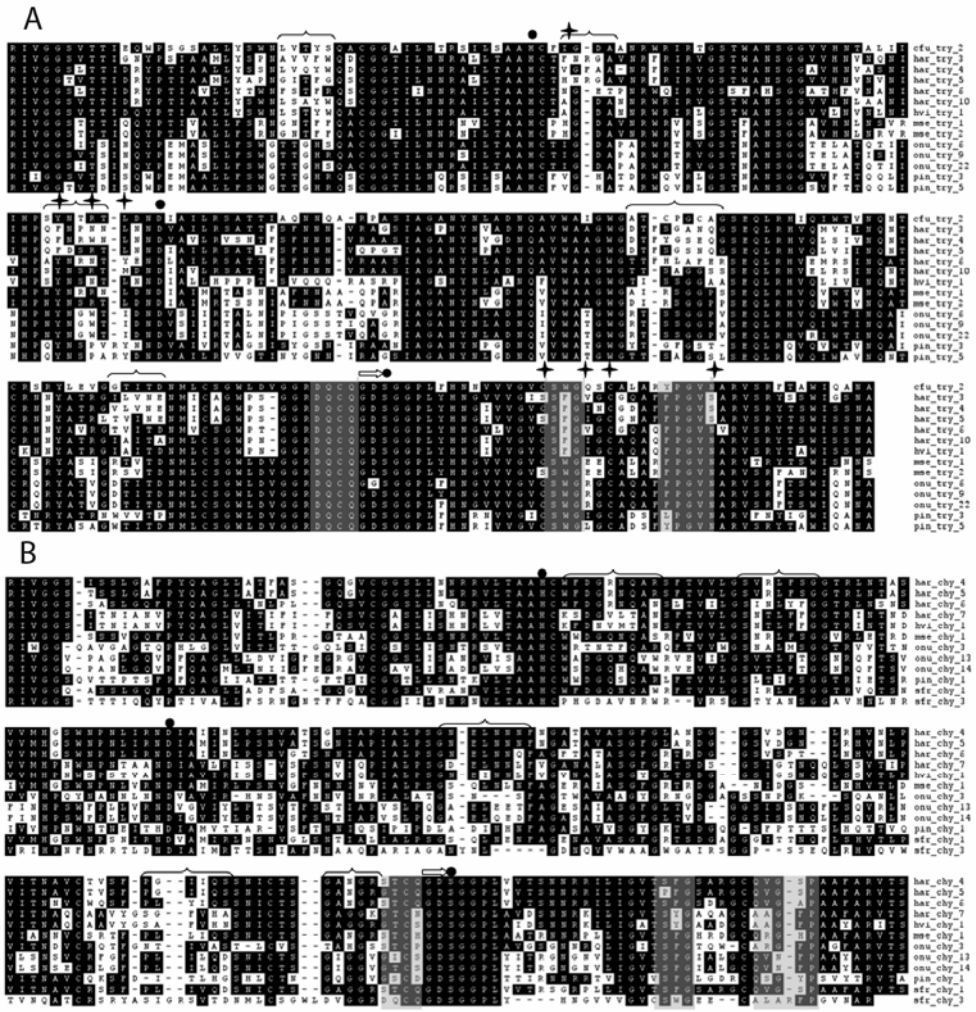

Fig. 2. Molecular diversity in Lepidopteran serine proteases. (A) Trypsins. (B) Chymotrypsins. Sequences of representative Lepidopteran trypsins and chymotrypsins were aligned by the Clustal algorithm to illustrate the occurrence of amino acid variations in the catalytic triad (●), oxyanion hole (⇒) and S1 pocket (grey), as well as in those regions putatively involved in the formation of adventitious contacts (horizontal parenthesis) and the 'hot-spots' which govern sensitivity to PIs (marked by '+'). Trypsin sequences are from *Choristoneura fumiferana* (cfu_try_2, AAA81525); *Helicoverpa armigera* (har_try_3, CAA72956; har_try_4, CAA72955; har_try_5, CAA72954; har_try_6, CAA72949; har_try_10, CAA72962); *Heliothis virescens* (hvi_try_1, AAF43708), *Manduca sexta* (mse_try_1, P35047; mse_try_2, P35046); *Ostrinia nubilalis* (onu_try_6, AAX62035; onu_try_9, AAX62032; onu_try_22, AAR98918,); *Plodia interpunctella* (pin_try_3, AAF24226; pin_try_5, AAC36248). Chymotrypsin sequences are from *H. armigera* (har_chy_4, CAA72960; har_chy_5, CAA72959; har_chy_6, CAA72958; har_chy_7, CAA72952); *H. virescens* (hvi_chy_1, AAF43709); *M. sexta* (mse_chy_1, AAA58743); *O. nubilalis* (onu_chy_3, AAX62030; onu_chy_13, AAX62027; onu_chy_14, AAX62026); *P. interpunctella* (pin_chy_1, AAC36149); *Spodoptera frugiperda* (sfr_chy_1, AA075039; sfr_chy_3, AAC36150).

adventitious contacts; not surprisingly, a high degree of variation is observed in these regions as well as in the 'hot spots' (indicated by a '+' in Fig. 2A), which determine (in)sensitivity to PIs. Experiments on synthetic polypeptide enzymes or 'pepzymes' [29] have provided initial clues on the alteration of the activity and/or specificity resulting from modifying key amino acids [30-37]. It does appear that we cannot yet predict functional properties based on structural features. However, a combination of theoretical and experimental studies are essential to derive algorithms that analyze the behavior of amino acids as a function of external conditions and/or micro-environments within the polypeptide. Simultaneously, simplified empirical rules may also be helpful within the defined scope of the amino acid type (hydrophobic, acidic, aromatic, etc.) at these specificity-determining sites. Adventitious contacts may, however, be trickier to decipher due to the amino acid diversity that is generally observed at these regions. Although empirical rules generally risk being invalidated by experimental evidence, such attempts would aid the refinement of the complex algorithms. A holistic approach is thus needed for predicting the structure-function relationship of newly identified protease genes for a better understanding of the complexities involved in their evolution, expression and regulation.

LIMITATIONS IN EXPERIMENTAL ANALYSES OF FUNCTIONAL DIVERSITY

As noted previously, theoretical and experimental approaches are equally vital for the functional characterization of polypeptides. Though theoretical means may be advantageous in multiple analyses of different structural variants, experimental approaches provide concrete evidence pertaining to activities. However, 'wet-lab' methods carry risks associated with reproducibility and reliability at all stages of research. The purity of the polypeptide under consideration is of utmost importance; although limited impurities can be tolerated under experimental conditions, these contaminants may cause hindrances, especially if they happen to be co-purified isoforms. Unfortunately, conventional means of purification possess limited sensitivity in dealing with protease isoforms; subtle changes in properties due to changes in a few amino acids are not easily exploitable properties for the detection and/or separation of isoforms. These problems are compounded by the limited flexibility offered by contemporary activity-based detection systems. Though standardized synthetic substrates are commercially available for assaying various protease specificities, minor changes due to amino acid alterations may not be detectable [38]. The differences in the profiles of digestive proteases observed between laboratory-reared insect larvae and field-collected larvae [3, 39] could restrict the identification of protease isoforms, which are expressed selectively under field conditions. Careful handling of crude or (semi-) purified protease preparations is necessary to minimize autolysis which otherwise may lead to a loss of minor

activities, and hence a failure in identifying multiple isoforms and/or specificities. If sequence information is available, it is possible to express putative protease gene products in heterologous systems like bacteria (e.g. *Escherichia coli*) or yeasts (e.g. *Pichia pastoris*) [40] to obtain a sufficient quantity of reasonably pure protein. However, the toxicity of the expressed protease to the host organism can hinder this approach. Misfolding of the recombinant polypeptide into an inactive conformational state is an acknowledged possibility especially in bacterial expression systems; yeast expression systems may be relatively more successful in dealing with proteins derived from higher organisms like plants. Misfolding may also give rise to the formation of 'inclusion bodies' – insoluble aggregates of the recombinant protein within the microbial cells – leading to cell-death and decreased yield. A prudent approach for heterologous expression of proteases would involve initial synthesis of the protease in an inactive ('pre-protein') form that may be activated later by *in vitro* enzymatic modification, e.g. by controlled proteolytic cleavage of the leader peptide on support matrices. However, cleavage at unexpected sites on the recombinant protein may still lead to a loss of activity in the recombinant protein. In spite of these difficulties, some success has been achieved in the case of carboxypeptidases, where a recombinant zymogen form was expressed and processed further *in vitro* to obtain the active form [41, 42], thus enabling researchers to gain useful information on novel insect gut carboxypeptidases [43]. Recent reports describe the stable and functional expression of serine proteases by use of insect cell culture systems and viral vectors for a novel chymotrypsin from *Spodoptera exigua* [44] and a serine protease from the firefly, *Pyrocelia rufa* [45]. These successful attempts will definitely spur more interest in expression systems based on insect cell culture and viral vector systems; a demand for specific cell-lines and customized vectors for various mechanistic classes of proteases also seems likely. However, simultaneous efforts for improving microbial systems for heterologous protease expression is essential, considering the relative ease in microbial culture as compared to use of insect cell lines. Difficulties arise in experimental set-ups for various reasons including the innate nature of the molecule under study and external factors associated intimately with the overall method. The innate properties of proteases that hinder handling cannot be changed but they can be temporarily bypassed by using physical/chemical agents during the study. Although it might not be possible to eliminate the external hindrances associated with factors such as sensitivity, they can definitely be limited by including checks based on our current understanding.

PROTEASES AS TARGET IN INSECT CONTROL PROGRAMS

Due to the importance of digestive proteases in larval physiology, plant derived PIs have received continuous attention [46-52]. Formation of the protease-PI complex yields a stable and inactive acyl-enzyme [53-56], resulting in distortion

of the active site (catalytic triad) of serine proteases. This loss of protease activity retards digestion and leads to crippling effects not only on larval growth and development [57-60] but also on the fertility and fecundity of the adult moths [50-65]. However, not all PIs may inhibit larval gut proteases due to absence of a specific protease-binding site and/or degradation by the insect gut proteases [66]. Altered binding capabilities in proteases may also lead to unexpected results, as exemplified by the inhibition of (predominantly trypsin-like) HGP activity by the winged bean (*Psophocarpus tetragonolobus*) Kunitz type chymotrypsin inhibitor [67], and the chickpea (*Cicer arietinum*) Kunitz type PI, which possesses an active site variation and was not expected to inhibit HGP-trypsins [60]. The diversity of digestive protease may also determine resistance to other molecules such as amylase inhibitors (AIs) and the *Bacillus thuringiensis* δ endotoxin (*Bt* toxin). It has been reported in the case of the Mexican bean weevil (*Zabrotes subfasciatus*) that resistance to the AIs of common bean (*Phaseolus vulgaris*) follows a two-pronged strategy [68] – the insect adapted by synthesizing not only AI-resistant amylases, but also employed proteases that effectively degraded and inactivated the AI protein. In case of *Bt*, resistance to the toxin may arise due to the absence of protease isoforms that recognize the pro-toxin and convert it to the toxic form [69]. Indeed, varying levels of midgut proteases [70-73] have been linked to resistance/susceptibility to *Bt*. Evidently, the feeding habits of the insect, with respect to its choice of host plant(s) as well as exposure and possible adaptation to PI(s) that lead to changes in gut proteolytic complement can be expected to play an important role in influencing susceptibility to *Bt* toxin and other PIs. This aspect cannot be overlooked while devising strategies for insect control that involve pyramiding of PI and *Bt* genes for host-plant transformation. Although the outcome of such gene-pyramiding on insect responses cannot be entirely predicted, it is possible to gain a lead by, once again, studying insect responses to varying dietary profiles (including PI-incorporation). Information on changes in gut proteolytic specificities with respect to *Bt* sensitivity/resistance may well be advantageous in designing tailor-made *Bt*-PI gene combinations for specific target crops. Though the signaling mechanism associated with the regulation of gut proteases is not well understood, it does not take much to figure out that this could be a further target for insect control strategies; such approaches would be based on the blocking of putative 'messenger' molecules that relay information between the brain and gut. The significance of Lepidopteran gut proteases as a critical target for insect control programs is clearly understood when considering the numerous processes that they are associated with. It is interesting to note that the digestive proteolytic machinery is linked not only with nutrient uptake and growth, but also with adaptation to newer hosts and resistance to antagonistic agents, to name a few. There could be other critical process linked to digestion that we are not currently aware of; perhaps these newer functions may also be exploitable for insect control in the future.

EVOLUTION AND ADAPTATION: LESSONS FROM THE PAST, BUT CAN WE FORESEE THE FUTURE?

As discussed previously, the extreme diversity in larval gut proteases often works out in favor of the insect. Coupled with an effective signaling mechanism, polyphagous larvae have an enviable ability to alter their digestive protease complement in response to change in the nutritional quality of the diet and/or towards nutritional challenge(s) arising due to ingestion of anti-metabolic agents like PIs; in many cases, insects are able to successfully escape the anti-metabolic effects of dietary PIs (Tab. 2). Faced with the prospect and consequences of reduced nutrient uptake, the larva responds by quantitatively and/or qualitatively

Tab. 2. Larval responses to PIs previously reported in the literature

Insect	PI type	Response	Reference
Helicoverpa armigera	SKTI PinII Aprotinin PinI	Upregulation of chymotrypsins and downregulation of trypsins	Bown *et al*., 1997; 2004; Gatehouse *et al.*, 1997
	CaKPI	Weak upregulation of all specificities	Srinivasan *et al.*, 2005b
	GnPI BGPI	Upregulation of trypsins and aminopeptidases	Chougule *et al.*, 2005
	WBPI	Downregulation of trypsins and chymotrypsins	
Heliothis virescens	Nicotiana leaf PIs	Synthesis of PI-insensitive trypsins	Brito *et al.*, 2001
Helicoverpa zea	SKTI PinII	Upregulation of trypsins	Broadway and Duffey, 1986
	SKTI	Adapted trypsins are insensitive to SKTI, BBI, PinII and MTI-II	Volpicella *et al.*, 2003
	SKTI	Increased gut proteolytic activity	Broadway, 1997
Spodoptera exigua	SKTI PinII	Upregulation of trypsins	Broadway and Duffey, 1986
	PinII	Upregulation of trypsins	Jongsma *et al.*, 1995
Spodoptera litura	BGPI		Telang *et al.*, 2003
Agrotis ipsilon	SKTI	Increased gut proteolytic activity	Broadway, 1997
		Synthesis of PI-insensitive trypsins, upregulation and downregulation of various chymotrypsins	Mazumdar-Leighton and Broadway, 2001a
Trichoplusia ni	SKTI	Increased gut proteolytic activity	Broadway, 1997
		Synthesis of PI-insensitive trypsins, upregulation and downregulation of various chymotrypsins	Mazumdar-Leighton and Broadway, 2001a

SKTI, soybean (Glycine max) Kunitz trypsin inhibitor; PinI/II, potato (Solanum tuberosum) protease inhibitor I/II; CaKPI, Cicer arietinum Kunitz proteinase inhibitor; GnPI, groundnut (Arachis hypogea) proteinase inhibitor; BGPI, bitter gourd (Momordica charantia) proteinase inhibitor; WBPI, winged bean (Psophocarpus tetragonolobus) proteinase inhibitor.

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altering the gut proteases to compensate the loss of protease activity due to PI binding [6, 74-76]. Quantitative changes include generalized or specific increases in the levels of gut proteases to attain optimal rate of protein digestion [2, 57, 77, 78]. Qualitative responses include synthesis of "insensitive" protease isoforms which the PI is unable to bind to and inhibit [1, 59, 79-82], or which have the ability to bind and degrade the PI [38, 66, 68, 83-85]. Though the insect has a choice of combining these adaptive responses, adaptation is achieved only if the insect successfully metabolizes the altered diet and continues normal growth and development. Thus, response always precedes adaptation, but the latter might not always be observed [86]. Just as it is difficult to predict the fate of ingested PIs, insect responses may also be unforeseeable due to dynamism and diversity in digestive proteases. The link between insect adaptability and diversity of digestive protease genes is an interesting study from the evolutionary point of view. It is possible that random changes were followed by natural selection, determined by host plant availability and/or PIs. Whether the insects were pre-adapted to PIs or whether adaptation was gained during evolution is still not clear. However, studies on relationships between Lepidoptera and their host plants [87] have proposed relevant insights on co-evolutionary processes by probing the role of plant secondary metabolites in determining patterns of herbivore infestation. In a parallel context, we can understand the role of PIs that contribute to differentiation between host and non-host plants. Adaptation to one particular type of PI by one species of insect could offer it a broader host range, i.e. all plants that produce related PIs, as observed experimentally [59]. Likewise, the insects that successfully adapt to one plant species are at an advantage to diversify further and colonize related plant species as well – in other words, steady evolutionary responses are the dominant factors in insect evolution and adaptation. The faster life cycle of insects, coupled with their ability to reproduce in large numbers, ensures a rich pool of genetic diversity resulting from mutations as well as DNA recombination events. Apparently, the effects of random mutations on population dynamics are amplified by the high reproductive rate. Perhaps adaptation to newer plant species was also influenced by a relative shortage of preferred host-plant material, which in turn came about due to high reproductive rates in insects and voracious foraging. Such a mechanism could explain the polyphagous nature of pests like *H. armigera*. Monophagous insects, on the other hand, probably might not have diversified owing to sufficient availability of the preferred host-plant derived food material and/or lower reproductive rates. As will be illustrated in a later section, certain insects exhibit a specific preference to a particular host (and host-plant PIs) and do not respond well to similar PIs from related plant species. Evolutionary aspects of defense are also observed in plants, as evident from the structural and functional diversity in defensive molecules like PIs. Though the diversity observed in PIs is not as vast as in insect proteases, it is observed that plant defenses (PIs) are not grossly overshadowed by herbivorous insects. Newer

types of inducible and multi-domain PIs with diverse protease specificities and activities are identified regularly; evidently plants also seem to have an option of producing broad-range as well as specific PIs, either of which may be activated in response to insect wounding. In spite of the rapid diversification observed in insect proteases, the slower evolutionary process in plant defenses seems capable of countering insect offences. Thus, the co-adaptive evolutionary race between plants and insects aims to compensate for each others' arsenal, albeit at different rates. This is evident from the mutational hot-spots that have been identified in plant PIs as well as in insect gut proteases. Though plant-insect interactions remain hard to predict owing to complexities, it might just be possible to predict the outcome of future mutational events in both classes of macromolecules, even though predicting their function would still be a perplexing problem.

CASE STUDIES ON INSECT ADAPTATION TO PI

Helicoverpa armigera – a general feeder

H. armigera, a polyphagous herbivorous pest, seems well adapted to most of the different mechanistic classes of plant PIs, thus can successful infest diverse crops like chickpea (*Cicer arietinum*), okra (*Abelmoschus esculentus*) and cotton (*Gossypium hirsutum*). Studies on dietary incorporation of non-host plant PIs have shown that *H. armigera* responds and adapts by altering the complement of gut serine proteases [2, 5, 57, 62, 63, 68]. A complex pattern of differential protease gene expression was observed between insects fed on protein-rich legume hosts (chickpea and pigeonpea) and other hosts (tomato, okra and cotton), with the latter showing a generalized over-expression of all proteases [5], indicating adaptation towards varied host plant proteins and highlighting the rich protease diversity and complex regulatory mechanisms that contribute to the polyphagous nature of the insect pest. The presence of such a sheer number and variety of protease genes makes it a very interesting example for dissection of the molecular basis of protease gene differential expression.

Manduca sexta – a specialist feeder

The larvae of *Manduca sexta* are facultative herbivores on Solaneceous plants like tobacco (*Nicotiana tobacum*), tomato (*Lycopersicon exculentum*), potato (*Solanum tuberosum*) and capsicum (*Capsicum annum*). The larvae of *M. sexta* are initially polyphagous, but when reared on Solaneceous foliage, they develop a strong preference for their host. However, feeding experiments showed that *M. sexta* larvae initially reared on untransformed tobacco leaves and then fed on transgenic tobacco leaf discs expressing tomato PIs exhibited a significant decrease in growth and development [88]. Larval adaptation towards one specific host plant or host-plant PI usually manifests in adaptation to other plants or host-plant PIs of the same family, as observed in case of *H. armigera*. However, adaptation of *M. sexta* to one host plant (and its PIs) does not impart any resistance to PIs derived from host plants of the same family or that belong

same group of (wound-inducible) serine PIs. It appears that polyphagy at the earlier larval stage helps the insect to survive with any available plant sources. Once it infests Solaneceous plants, the larvae can easily feed on these plants with a basic set of proteases without any stress on its digestive system arising due to plant PIs. While this loss of flexibility with respect to host range may have fitness benefits in maintaining a lower degree of complexity in the synthesis of gut proteases, it probably also takes away the adaptability in the case of a sudden change in host.

***Spodoptera exigua* – a general feeder**

The beet armyworm *Spodoptera exigua* is a widespread polyphagous pest causing severe economic damage to crops like beet (*Beta vulgaris*), cowpea (*Vigna unguiculata*) and eggplant (*Solanum melongena*). When challenged with the barley trypsin inhibitor BTI-CMe [89], *S. exigua* larvae responded by increasing the expression of leucine-aminopeptidase and carboxypeptidase A-like proteinase. As a direct result of TI ingestion, gut-trypsin activity was reduced but chymotrypsin-, elastase- and carboxypeptidase B-like activities were not affected. The distinctive feature of this response is a downregulation of the dominant mechanistic class of endopeptidase involved in primary digestion (trypsin) and simultaneous uregulation of exopeptidases usually associated with secondary digestion (amino- and carboxypeptidases). Though changes in the expression of exopeptidases have been reported in response to serine PIs [41], their significance is not very clear. It would seem that the exopeptidases that would degrade the PI (BTI-CMe) have a much greater role in protein digestion than currently understood.

Analyses of the three different types of larval response, i.e. (i) adaptation to multiple hosts by diversity in the major gut protease component (*H. armigera*), (ii) adaptation to a single host by use of a specific or narrow choice of gut proteases (*M. sexta*), and (iii) adaptation to hosts or PIs by use of diverse mechanistic classes (*S. exigua*), revealed the complexities in the digestive, response and adaptive processes in insects. Such studies on various other mechanisms may aid in the prediction of insect adaptive responses. Although it may not be possible to define rules of thumb for every insect, a case-to-case study would go a long way in predicting generalized insect responses. Though the relationship of insect responses to dietary input, i.e. host-plant preference(s), has not been established, some insight has been obtained by way of *in vivo* studies [5]. Further studies are necessary to probe and establish such links based on conclusive evidence; this could be an area of strong potential to model further research. The identification and functional characterization of digestive proteases and the deciphering of the regulatory elements and processes is of paramount importance and should proceed in parallel with host-pest case studies. Such studies could prove invaluable in devising “custom-defense strategies” for crop plants, and these tailor-made approaches are essential to ensure crop survival even under extreme threat of insect attack.

SUMMARY

Though the contribution of gut proteases to digestion and other key functions in insects is a well-researched topic, some questions remain unanswered. The existence of microbial flora in the insect gut has not been ruled out, but their presence and contribution to digestive processes has not yet been proven unambiguously. Considering the significance of bacteria and other microbes in higher animals, any insight into the existence of similar symbiotic systems in insects would be a significant discovery with respect to the physiological processes linked to gut activities. Likewise, limited information is available on the actual basis for the regulation of gut protease gene expression. When considering differential expression involving multiple mechanistic classes, our understanding falls behind. The concrete relationships between the structural and functional aspects of proteases have not been established. Current understanding of the dynamic nature of the Lepidopteran digestive proteases is vast, but insufficient for the development of "fool-proof" strategies for insect control. A thorough understanding of the mechanisms and signaling pathways governing the digestive processes in insect pests is thus necessitated. Although it will be a while before significant insights are achieved, currently available information suggests that it would be possible to control insect-mediated damage and stem losses if not totally stop infestation. Perhaps the biggest threat to agriculture may not be insect attack, but rather limitations on our understanding of the insect pest, which lead to improper strategies in insect control. In the long run, such ill-devised approaches risk becoming ineffective against the insect pest for the simple reason of insect resistance. Natural plant defenses are also unknowingly ignored due to modern agricultural practices like selection criteria, mono-culturing of crop plants, and uses of chemical pesticides. Though the balance seems tilted in favor of insect pests at present, future studies could eventually help us to control, if not to overcome the devastating effects of insect herbivory.

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REFERENCES

1. Bown, D.P., Wilkinson, H.S. and Gatehouse, J.A. Differentially regulated inhibitor sensitive and insensitive protease genes from the phytophagous pest, *Helicoverpa armigera*, are members of complex multigene families. **Insect Biochem. Mol. Biol.** 27 (1997) 625-638.

2. Gatehouse, L.N., Shannon, A.L., Burgess, E.P.J. and Christeller, J.T. Characterization of major midgut proteinase cDNAs from *Helicoverpa armigera* larvae and changes in gene expression in response to four proteinase inhibitors in the diet. **Insect Biochem. Mol. Biol.** 27 (1997) 929-944.
3. Patankar, A.G., Giri, A.P., Harsulkar, A.M., Sainani, M.N., Deshpande, V.V., Ranjekar, P.K. and Gupta, V.S. Complexity in specificities and expression of *Helicoverpa armigera* gut proteinases explains polyphagous nature of the insect pest. **Insect Biochem. Mol. Biol.** 31 (2001) 453-464.
4. Browne, L.B. and Raubenheimer, D. Ontogenic changes in the rate of ingestion and estimates of food consumption in fourth and fifth instar *Helicoverpa armigera* caterpillars. **J. Insect Physiol.** 49 (2003) 63-71.
5. Chougule, N.P., Giri, A.P., Sainani, M.N. and Gupta, V.S. Gene expression patterns of *Helicoverpa armigera* gut proteases. **Insect Biochem. Mol. Biol.** 35 (2005) 355-367.
6. Bown, D.P., Wilkinson, H.S. and Gatehouse, J.A. Regulation of expression of genes encoding digestive proteases in the gut of a polyphagous lepidopteran larva in response to dietary protease inhibitors. **Physiol. Entomol.** 29 (2004), 278-290.
7. Huang, Y., Brown, M.R., Lee, T.D. and Crim, J.W. RF-amides isolated from the midgut of the corn earworm *Helicoverpa zea*, resemble pancreatic polypeptide. **Insect Biochem. Mol. Biol.** 28 (1998) 345-356.
8. Harshini, S., Nachman, R.J. and Sreekumar, S. In vitro release of digestive enzymes by FMRF amide related neuropeptides and analogues in the lepidopteran insect *Opisina arenosella* (Walk.) **Peptides** 23 (2002) 1759-1763.
9. Harshini, S., Nachman, R.J. and Sreekumar, S. Inhibition of digestive enzyme release by neuropeptides in larvae of *Opisina arenosella* (Lepidoptera: Cryptophasidae). **Comp. Biochem. Physiol**. **- Part B** 132 (2002) 353-358.
10. Lopes, A.R., Juliano, M.A., Juliano, L. and Terra, W.R. Coevolution of insect trypsins and inhibitors. **Arch. Insect Biochem. Physiol.** 55 (2004) 140-152.
11. Barrett, A.J. and Rawlings, N.D. Families and clans in serine peptidases. **Arch. Biochem. Biophys.** 318 (1995) 247-250.
12. Krem, M.M. and Cera E.D. Molecular markers of serine protease evolution. **EMBO J.** 20 (2001) 3036-3045
13. Hegedus, D., Baldwin, D., O'Grady, M., Braun, L., Gleddie, S., Sharpe, A., Lydiate, D. and Erlandson, M. Midgut proteases from *Mamestra configurata* (Lepidoptera: Noctuidae) larvae: characterization, cDNA cloning and expressed sequence tag analysis. **Arch. Insect Biochem. Physiol.** 53 (2003) 30-47.

14. Botos, I., Meyer, E., Nguyen, M., Swanson, S.M., Koomen, J.M., Russell, D.H. and Meyer, E.F., 2000. The structure of an insect chymotrypsin. **J. Mol. Biol.** 298 (2000) 895-901.
15. Iengar, P. and Ramakrishnan, C. Knowledge based modeling of the serine protease triad into non-proteases. **Protein Eng.** 12 (1999) 649-655.
16. Nishihira, J. and Tachikawa, H. Theoretical evaluation of a model of the catalytic triads of serine and cysteine proteases by an initio molecular orbital calculation. **J. Theor. Biol.** 196 (1999) 513-519.
17. Beveridge, A.J. A theoretical study of the initial stages of catalysis in the aspartic proteinases. **J. Mol. Chem. (Theochem)** 453 (1998) 275-291.
18. Laskowski, M. Jr, and Qasim, M.A. What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes? **Biochim. Biophys. Acta** 1477 (2000) 324-337.
19. Krowarsch, D., Zakrzewska, M., Smalas, O.A. and Otlewski, J. Structure-function relationships in serine protease-bovine pancreatic trypsin inhibitor interaction. **Protein Pept. Lett.** 12 (2005) 1-5.
20. Kraut, J. Serine proteases: structure and mechanism of catalysis. **Annu. Rev. Biochem.** 46 (1977) 331-358.
21. Dodson, G. and Wlodawer, A. Catalytic triad and their relatives. **Trends Biochem. Sci.** 23 (1998) 347-352.
22. Polgar, L. The catalytic triad of serine peptidases. **Cell. Mol. Life Sci.** 62 (2005) 1-12.
23. Hunkapiller, M.W., Smallcombe, S.H., Hitaker, D.R. and Richards, J.H. Ionization behaviour of the histidine residue in the catalytic triad of serine proteases. **J. Biol. Chem.** 248 (1973) 8306-8308.
24. David, F., Bernard, A.M., Pierres, M. and Marguet, D. Identification of serine 624, aspartic acid 702 and histidine 734 as the catalytic triad residues of mouse dipeptidyl-peptidase IV (CD26): a member of the novel family of nonclassical serine hydrolases. **J. Biol. Chem.** 268 (1993) 17247-17252.
25. Ishida, T. and Kato, S. Role of asp102 in the catalytic relay system of serine proteases: a theoretical study. **J. Am. Chem. Soc.** 126 (2004) 7111-7118.
26. Komiyama, T., VanderLugt, B., Fugere, M., Day, R., Kaufman, R.J. and Fuller, R.S. Optimization of protease-inhibitor interactions by randomizing adventitious contacts. **Proc. Nat. Acad. Sci. USA** 100 (2003) 8205-8210.
27. Marana, S.R., Lopes, A.R., Juliano, L., Juliano, M.A., Ferreira, C. and Terra, W.R. Subsites of trypsin active site favor catalysis or substrate binding. **Biochem. Biophys. Res. Comm.** 290 (2002) 494-497.
28. Fodor, K., Harmat, V., Hetenyi, C., Kardos, J., Antal, J., Perczel, A., Patthy, A., Katona, G. and Graf, L. Extended intermolecular interactions in a serine protease canonical inhibitor complex account for strong and highly specific inhibition. **J. Mol. Biol.** 350 (2005) 156-169.
29. Atassi, M.Z., Manshouri, T. Design of peptide enzymes (pepzymes): Surface-simulation synthetic peptides that mimic the chymotrypsin and

trypsin active sites exhibit the activity and specificity of the respective enzyme. **Proc. Natl. Acad. Sci. USA.** 90 (1993) 8282-8286.
30. Kaiser, E.T., Lawrence, D.S. and Rokita, S.E. The chemical modification of enzyme specificity. **Annu. Rev. Biochem.** 54 (1985) 565-595.
31. El-Hawrani, A.S., Sessions, R.B., Moreton, K.M. and Holbrook, J.J. Guided evolution of enzymes with new substrate specificities. **J. Mol. Biol.** 264 (1996) 97-110.
32. Hung, S. and Hedstrom, L. Converting trypsin to elastase: substitution of the S1 site and adjacent loops reconstitutes esterase specificity but not amidase activity. **Protein Eng.** 11 (1998) 669-673.
33. Takagi, H. and Takahashi, M. A new approach for alteration of protease functions: pro-sequence engineering. **Appl. Microbiol. Biotechnol.** 63 (2003) 1-9.
34. Khamrui, S., Dasgupta, J., Dattagupta, J.K. and Sen, U. Single mutation at P1 of a chymotrypsin inhibitor changes it to a trypsin inhibitor: X-ray structural (2.15°A) and biochemical basis. **Biochim. Biophys. Acta** (2005) in press.
35. Corey, D.R., Willett, W.S., Coombs, G.S. and Craik, C.S. Trypsin Specificity Increased through Substrate-Assisted Catalysis. **Biochemistry** 34 (1995) 11521-11527.
36. Higaki, J.N., Evnin, L.B. and Craik, C.S. Introduction of a Cysteine Protease Active Site into Trypsin. **Biochemistry** 28 (1989) 9256-9263.
37. Tanaka, T. and Yada, R.Y. Redesign of catalytic center of an enzyme: aspartic to serine proteinase. **Biochem. Biophys. Res. Commun.** 323 (2004) 947-953.
38. Telang, M.A., Giri, A.P., Sainani, M.N. and Gupta, V.S. Elastase-like proteinase of *Helicoverpa armigera* is responsible for inactivation of a proteinase inhibitor from chickpea. **J. Insect Physiol.** 51 (2005) 513-522.
39. Valaitis, A.P., Augustin, S. and Clancy, K.M. Purification and characterization of the western spruce budworm larval midgut proteinases and comparison of gut activities of laboratory-reared and field-collected insects. **Insect Biochem. Mol. Biol.** 29 (1999) 405-415.
40. Bown, D.P., Wilkinson, H.S., Jongsma, M.A. and Gatehouse, J.A. Characterization of cysteine proteinases responsible for digestive proteolysis in guts of larval western corn rootworm (*Diabroticca virgifera*) by expression in the yeast *Pichia pastoris*. **Insect Biochem. Mol. Biol.** 34 (2004) 305-320.
41. Bown, D.P., Wilkinson, H.S. and Gatehouse, J.A. Midgut carboxypeptidase from *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae: enzyme characterisation, cDNA cloning and expression. **Insect Biochem. Mol. Biol.** 28 (1998) 739-749.
42. Bayes, A., Sonnenschein, A., Daura, X., Vendrell, J. and Aviles, F.X. Procarboxypeptidase A from the insect pest *Helicoverpa armigera* and its derived enzyme. **Eur. J. Biochem.** 270 (2003) 3026-3035.

43. Estebanez-Perpina, E., Bayes, A., Vendrell, J., Jongsma, M.A., Bown, D.P., Gatehouse, J.A., Huber, R., Bode, W., Aviles, F.X. and Reverter, D. Crystal structure of a novel mid-gut procarboxypeptidase from the cotton pest *Helicoverpa armigera*. **J. Mol. Biol.** 313 (2001) 629-638.
44. Herrero, S., Combes, E., Van Oers, M.M., Vlak, J.M., de Maagd, R.A. and Beekwilder, J. Identification and recombinant expression of a novel chymotrypsin from *Spodoptera exigua*. **Insect Biochem. Mol. Biol.** 35 (2005) 1073-1082.
45. Li, J., Choo, Y.M., Lee, K.S., Je, Y.H., Woo, S.D., Kim, I., Sohn, H.D. and Jin, B.R. A serine protease gene from the firefly, *Pyrocoelia rufa*: gene structure, expression, and enzyme activity. **Biotechnol. Lett.** 27 (2005) 1051-1057.
46. Garcia-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, L., Roys, J. and Carbonero, P. Plant proteinaceous inhibitors of proteases and amylases. **Oxford Survey Plant Mol. Cell. Biol.** 4 (1987) 275-334.
47. Ryan, C.A. Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. **Annu. Rev. Phytopathol.** 28 (1990) 425-449.
48. Boulter, D. Insect pest control by copying nature using genetically engineered crops. **Phytochemistry** 34 (1993) 1453-1466.
49. Carlini, C.R. and Grossi-de-Sa, M.F. Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. **Toxicon** 40 (2002) 1515-1539.
50. Murdock, L.L. and Shade, R.E. Lectins and protease inhibitors as plant defenses against insects. **J. Agric. Food Chem.** 50 (2002) 6605-6611.
51. Ferry. N., Edwards, M.G., Gatehouse, J.A. and Gatehouse, A.M.R. Plant-insect interactions: molecular approaches to insect resistance. **Curr. Opin. Biotechnol.** 15 (2004) 155-161.
52. Giri, A.P., Chougule, N.P., Telang M.A. and Gupta, V.S. Engineering insect tolerant plants using plant defensive proteinase inhibitors. in: **Recent Research Developments in Phytochemistry**, (Pandalai, S.G., Ed) Research Signpost, India, vol. 8, 2005, 117-137.
53. Laskowski, M. Jr. Protein inhibitors of serine proteinases – mechanism and function. **Adv. Exp. Med. Biol.** 199 (1986) 1-17.
54. Huntington, J.A., Read, R.J. and Carrell, R.W. Structure of a serpin–protease complex shows inhibition by deformation. **Nature** 407 (2000) 923-926.
55. Hedstrom, L. Serine protease mechanism and specificity. **Chem. Rev.** 102 (2000) 4501-4523.
56. Plotnick, M.I., Mayne, L., Schechter, N.M. and Harvey, R. Distortion of the Active Site of Chymotrypsin Complexed with a Serpin. **Biochemistry** 35 (1996) 7586-7590.
57. Broadway, R.M. and Duffey, S.S. Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. **J. Insect Physiol.** 32 (1986) 827-833.

58. Hilder, V.A., Gatehouse, A.M.R., Sherman, S.F., Barker, R.F. and Boulter, D. A novel mechanism of insect resistance engineered into tobacco. **Nature** 330 (1987) 160-163.
59. Broadway, R.M. Plant dietary proteinase inhibitors alter complement of midgut proteases. **Arch. Insect Biochem. Physiol.** 32 (1996) 39-53.
60. Srinivasan, A., Giri, A.P., Harsulkar, A.M., Gatehouse, J.A. and Gupta, V.S. A Kunitz trypsin inhibitor from chickpea (*Cicer arietinum* L.) that exerts anti-metabolic effect on podborer (*Helicoverpa armigera*) larvae. **Plant Mol. Biol.** 57 (2005) 359-374.
61. Jouanin, L., Bonade-Bottino, M., Girard, C., Morrot, G and Giband, M. Transgenic plants for insect resistance. **Plant Sci.** 131 (1998) 1-11.
62. Harsulkar, A.M., Giri, A.P., Patankar, A.G., Gupta, V.S., Sainani, M.N., Ranjekar, P.K. and Deshpande, V.V. Successive use of non-host plant proteinase inhibitors required for effective inhibition of *Helicoverpa armigera* gut proteinases and larval growth. **Plant Physiol.** 121 (1999) 497-506.
63. deLeo, F. and Gallerani, R. The mustard trypsin inhibitor 2 affects the fertility of *Spodoptera littoralis* larvae fed on transgenic plants. **Insect Biochem. Mol. Biol.** 32 (2002) 489-496.
64. Telang, M.A., Srinivasan, A., Patankar, A.G., Harsulkar, A.M., Joshi, V.V., Damle, A., Deshpande, V.V., Sainani, M.N., Ranjekar, P.K., Gupta, G.P., Birah, A., Rani, S., Kachole, M., Giri, A.P and Gupta, V.S. Bitter gourd proteinase inhibitors: potential growth inhibitors of *Helicoverpa armigera* and *Spodoptera litura*. **Phytochemistry** 63 (2003) 643-652.
65. Tamhane, V.A., Chougule, N.P., Giri, A.P., Dixit, A.R., Sainani, M.N. and Gupta, V.S. *In vitro* and *in vivo* effects of *Capsicum annum* proteinase inhibitors on *Helicoverpa armigera* gut proteinases. **Biochim. Biophys. Acta** 1722 (2005) 155-167.
66. Giri, A.P., Harsulkar, A.M., Deshpande, V.V., Sainani, M.N., Gupta, V.S. and Ranjekar, P.K. Chickpea defensive proteinase inhibitors can be inactivated by podborer gut proteinases. **Plant Physiol.** 116 (1998) 393-401.
67. Giri, A.P., Harsulkar, A.M., Ku, M.S.B., Gupta, V.S., Deshpande, V.V., Ranjekar, P.K. and Franceschi, V.R. Identification of potent inhibitors of *Helicoverpa armigera* gut proteinases from winged bean seeds. **Phytochemistry** 63 (2003) 523-532.
68. Ishimoto, M. and Chrispeels, M.J. Protective mechanism of the Mexican bean weevil against high levels of α-amylase inhibitor in the common bean. **Plant Physiol.** 111 (1996) 393-401.
69. Oppert, B., Kramer, K.J., Johnson, D., Upton, S.J. and McGaughey, W.H. Luminal proteinases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* Cry1A(c) protoxin. **Insect Biochem. Mol. Biol.** 26 (1996) 571-583.

70. Zhu, Y., Oppert, B., Kramer, K.J., McGaughey, W.H. and Dowdy, A.K. cDNAs for a chymotrypsinogen-like protein from two strains of *Plodia interpunctella*. **Insect Biochem. Mol. Biol.** 27 (1997) 1027-1037.
71. Zhu, Y., Kramer, K.J., Oppert, B. and Dowdy, A.K. cDNAs of aminopeptidase-like protein genes from *Plodia interpunctella* strains with different susceptibilities to *Bacillus thuringiensis* toxins. **Insect Biochem. Mol. Biol.** 30 (2000) 215-224.
72. Zhu, Y., Kramer, K.J., Dowdy, A.K. and Baker, J.E. Trypsinogen-like cDNAs and quantitative analysis of mRNA levels from the indianmeal moth, *Plodia interpunctella*. **Insect Biochem. Mol. Biol.** 30 (2000) 1027-1035.
73. Zhu, Y., Oppert, B., Kramer, K.J., McGaughey, W.H. and Dowdy, A.K. cDNA sequence, mRNA expression and genomic DNA of trypsinogen from the indianmeal moth *Plodia interpunctella*. **Insect Mol. Biol.** 9 (2000) 19-26.
74. Jongsma, M.A. and Bolter, C. The adaptation of insects to plant protease inhibitors. **J. Insect Physiol.** 43 (1997) 885-895.
75. Paulillo, L.C.M.S., Lopes, A.R., Cristofoletti, P.T., Parra, J.R.P., Terra, W.R. and Silva-Filho, M.C. Changes in midgut endopeptidase activity of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) are responsible for adaptation to soybean proteinase inhibitors. **J. Econ. Entomol.** 93 (2000) 892-896.
76. Brito, L.O., Lopes, A.R., Parra, J.R.P., Terra, W.R. and Silva-Filho, M.C. Adaptation of tobacco budworm *Heliothis virescens* to proteinase inhibitors may be mediated by the synthesis of new proteinases. **Comp. Biochem. Physiol.** 128 (2001) 365-375.
77. Broadway, R.M. Dietary regulation of serine proteinases that are resistant to serine proteinase inhibitors. **J. Insect Physiol.** 43 (1997) 855-874.
78. Girard, C., Metayer, M.L., Zaccomer, B., Bartlet, E., Williams, I., Bonade-Bottino, M., Pham-Delegue, M. and Jouanin, L. Growth simulation of beetle larvae reared on a transgenic oilseed rape expressing a cysteine proteinase inhibitor. **J. Insect Physiol.** 44 (1998) 263-270.
79. Jongsma, M.A., Bakker, P.L., Peters, J., Bosch, D. and Stiekema, W.J. Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. **Proc. Nat. Acad. Sci. USA** 92 (1995) 8041-8045.
80. Mazumdar-Leighton, S. and Broadway, R.M. Identification of six chymotrypsin cDNAs from larval midguts of *Helicoverpa zea* and *Agrotis ipsilon* feeding on the soybean (Kunitz) trypsin inhibitor. **Insect Biochem. Mol. Biol.** 31 (2001) 633-644.
81. Mazumdar-Leighton, S. and Broadway, R.M. Transcriptional induction of diverse midgut trypsins in larval *Agrotis ipsilon* and *Helicoverpa zea* feeding on the soybean trypsin inhibitor. **Insect Biochem. Mol. Biol.** 31 (2001) 645-657.

82. Volpicella, M., Ceci, L.R., Cordewener, J., America, T., Gallerani, R., Bode, W., Jongsma, M.A. and Beekwilder, J. Properties of purified gut trypsin from *Helicoverpa zea* adapted to proteinase inhibitors. **Eur. J. Biochem.** 270 (2003) 10-19.
83. Girard, C., Metayer, M.L., Bonade-Bottino, M., Pham-Delegue, M. and Jouanin, L. High level of resistance to proteinase inhibitors may be conferred by proteolytic cleavage in beetle larvae. **Insect Biochem. Mol. Biol.** 28 (1998) 229-137.
84. Zhu-Salzman, K., Koiwa, H., Salzman, R.A., Shade, R.E. and Ahn, J.E. Cowpea bruchid *Callosobruchus maculatus* uses a three-component strategy to overcome a plant defensive cysteine protease inhibitor. **Insect Biochem. Mol. Biol.** 12 (2003) 135-145.
85. Moon, J., Salzman, R.A., Ahn, J.E., Koiwa, H. and Zhu-Salzman, K. Transcriptional regulation in cowpea bruchid guts during adaptation to a plant defence protease inhibitor. **Insect Biochem. Mol. Biol.** 13 (2004) 283-291.
86. Srinivasan, A., Chougule, N.P., Giri, A.P., Gatehouse, J.A. and Gupta, V.S. Podborer (*Helicoverpa armigera* Hübn.) does not show specific adaptations in gut proteinases to dietary *Cicer arietinum* Kunitz proteinase inhibitor. **J. Insect Physiol.** 51 (2005) 1268-1276.
87. Ehrlich, P.R. and Raven P.H. Butterflies and plants: a study in coevolution. **Evolution** 18 (1964) 586-608.
88. Johnson, R., Narvaez, J., An, G. and Ryan, C.A. Expression of proteinase inhibitors I and II in transgenic tobacco plants: Effects on natural defense against *Manduca sexta* larvae. **Proc. Natl. Acad. Sci. USA** 86 (1989) 9871-9875.
89. Lara, P., Ortego, F., Gonzalez-Hidalgo, E., Castanera, P., Carbonero, P. and Diaz, I. Adaptation of *Spodoptera exigua* (Lepidoptera: Noctuidae) to barley trypsin inhibitor BTI-CMe expressed in transgenic tobacco. **Transgenic Res.** 9 (2000) 169-178.