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Differential antibiosis against *Helicoverpa armigera* exerted by distinct inhibitory repeat domains of *Capsicum annuum* proteinase inhibitors

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ABSTRACT

Plant defensive serine proteinase inhibitors (PIs) are known to have negative impact on digestive physiology of herbivore insects and thus have a crucial role in plant protection. Here, we have assessed the efficacy and specificity of three previously characterized inhibitory repeat domain (IRD) variants from *Capsicum annuum* PIs *viz*., IRD-7, -9 and -12 against gut proteinases from *Helicoverpa armigera*. Comparative study of *in silico* binding energy revealed that IRD-9 possesses higher affinity towards *H. armigera* serine proteinases as compared to IRD-7 and -12. *H. armigera* fed on artificial diet containing 5 TIU/g of recombinant IRD proteins exhibited differential effects on larval growth, survival rate and other nutritional parameters. Major digestive gut trypsin and chymotrypsin genes were down regulated in the IRD fed larvae, while few of them were up-regulated, this indicate alterations in insect digestive physiology. The results corroborated with proteinase activity assays and zymography. These findings suggest that the sequence variations among PIs reflect in their efficacy against proteinases *in vitro* and *in vivo*, which also could be used for developing tailor-made multi-domain inhibitor gene(s).

Introduction

There are many abiotic and biotic stresses affecting the crop productivity worldwide. *Helicoverpa armigera* and other Lepidopteron insect's infestation represents an important biotic factor that adversely affects the crops productivity (Ferry et al., 2004; Sharma et al., 2000). *H. armigera* is a polyphagous insect and easily adapts on various plants to obtain required nutrition by flexibility in expression of gut serine proteases namely, trypsin and chymotrypsin (Srinivasan et al., 2006). Furthermore, evolution of resistance mechanism has made pest management even more challenging (Tabashnik et al., 2008). In insects, serine proteases are involved in vital processes (digestion, metamorphosis and molting etc.) and inhibition of these processes may help to reduce crop damage and enhance productivity. Numerous efforts have been undertaken, with some still underway, to develop effective strategy of pest management by proteinase inhibitors (PIs) as antibiosis agents (Bown et al., 2004; Chougule et al., 2005; Christeller et al., 1992; Ryan, 1990). Thus, exploration, design and application of novel multi domain PI molecules targeting insect serine proteases are critical for effective control of *H. armigera.*

Overexpression of PIs, serves as an important tool for developing insect resistant plants (Johnson et al., 1989; Dunse et al., 2010a). However, evolution of adaptive mechanisms has facilitated herbivore insects to overcome the negative effects of PIs (de Oliveira et al., 2013; Dunse et al., 2010b). Potato inhibitor-II (Pin-II) is well studied as a defense molecule against plant pathogens, pests and nematodes (Turra and Lorito, 2011). Precursor of Pin-II PI protein consists of a series of disordered loop domains, which undergo proteolytic activation to form(s) inhibitory repeat domains (IRDs). Typical structure of IRD consists of 50 aa including eight cysteine and a single proline residues conserved throughout the population (Scanlon et al., 1999; Schirra and Craik, 2005). A part of IRD that interacts with target proteases is called as reactive site loop (RSL) and is found to be highly variable. RSL has coevolved with their target proteases, indicating its crucial role in plant-insect coevolution (Jongsma and Beekwilder, 2011). Conserved cysteine residues form a network of disulfide bonds and stabilize the repeat structure. Pin-II IRD variants with substituted cysteine residues might provide variable responses as compared to wild type (Joshi et al., 2014; Schirra et al., 2010). Variation in IRD sequences results in deviation in their stability and activity, thus this phenomenon can be explored for engineering effective inhibitor molecules against *H. armigera* gut proteases (Joshi et al., 2013).

Abbreviations: aa, amino acid; AD, artificial diet; CanPIs, *Capsicum annuum* proteinase inhibitors; HGP, *H. armigera* gut proteases; HaChy, *H. armigera* chymotrypsin; HaTry, *H. armigera* trypsin; IRD, inhibitory repeat domain; PIs, proteinase inhibitors.

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In our recent report, three IRDs namely, IRD-7, -9 and -12 were cloned and characterized for their structural and functional attributes. These findings indicate that IRD-9 exhibits enhanced protease inhibition due to lack of disulfide bond and flexibility in reactive loop as compared other IRDs (Joshi et al., 2014).

Here, we have assessed three previously identified IRDs from *C. annuum* PIs (CanPIs) using *in silico* and *in vitro* studies to understand inhibitory specificities of these inhibitors against trypsin, chymotrypsin, elastase and cathepsin-like proteases. *In vivo* efficacy of IRD-7, -9 and -12 was analyzed by monitoring growth performance and data derived from nutritional parameters. Response of insect digestive proteinases after ingestion of inhibitor was evaluated by proteinase gene expression, activity and zymography studies. This report demonstrates the approach of exploring sequence variations in proteinase inhibitors for designing a potent inhibitor for effective control of pests.

Result and discussion

In vitro assay indicates broad specificity and variable inhibition of various proteases activity by selected IRD variants

Sequence alignment of cloned inhibitors represented conserved cysteine residue position, while reactive site loop was the most variable region in the sequence (Joshi et al., 2014). Selected inhibitors demonstrated differential reactivity and specificity against various proteases. IRD-9 and -12 exhibited strong inhibition (70–90%) of trypsin, chymotrypsin and HGP, while IRD-7 (40–70%) showed comparatively less inhibition (Fig. 1). IRD-9 and -12 inhibited 30–50% of cathepsin and elastase activity, whereas IRD-7 showed 10–20% inhibition. It was observed that IRD-9 and IRD-12 displayed strong inhibitory effect on most of the proteinases examined. Activity assay against various proteases indicated that sequence variation in the IRDs might affect its inhibitory efficiency and target specificity. Although the selected IRDs were primarily trypsin inhibitors, they exhibited activity against chymotrypsin, elastase and cathepsin.

IRDs exhibit strong binding efficiency with various proteases

Docking and relative analysis displayed significant differences in binding energies suggesting that varied IRDs had variable interaction with *H. armigera* proteases. Among the three IRDs, IRD-9 shows strong interaction with the lowest binding energy with various *H. armigera* proteases (Fig. 2). As evident from activity inhibition assays, docking studies revealed broad specificity of IRDs with chymotrypsins, cathepsins and other serine proteases. Strong binding of IRD-9 with trypsin and chymotrypsin among all the three IRDs motivated us to compare *in vivo* effect of the recombinant IRD proteins against *H. armigera* digestive physiology. Interaction pattern of IRD-7 and -12 with most of the proteases was similar, which led to their clustering together for all the analyzed proteases. Binding energy comparison and hierarchical clustering analysis provides wide overview of specific interaction of inhibitor with various proteases. Furthermore, it will also give speculation about mode of action and effect of various inhibitors on *H. armigera* digestive proteases.

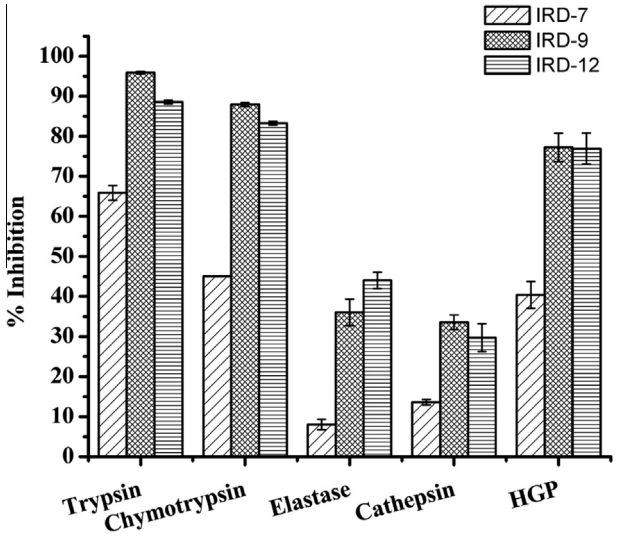

Fig. 1. Inhibition of bovine trypsin, bovine chymotrypsin, elastase, cathepsin and HGP by 20 μg of IRDs in azo-caseinolytic assays. Each value is an average of six replicates IRD-9 and -12 showed significant inhibitions in all different proteases than that of IRD-7 against all proteases.

IRDs retard the growth and development of H. armigera larvae

To understand the *in vivo* effect of IRDs on the development of *H. armigera* larvae, feeding experiments were conducted with appropriate controls. Active recombinant IRD proteins (5 TIU/g diet) were incorporated into diet to examine their *in vivo* potential against *H. armigera.* Development of larvae reared on a control and IRD protein-containing diets is presented in Fig. 3A. Feeding of insects on IRD-containing diet caused reduction in larval mass gain and survival rate. On day 11, larvae fed on diets containing IRD-9 and -12 weighed ~40% and 35% less, respectively, than the control (fed on AD without IRD-protein) larvae. In comparison, larvae fed on artificial diet containing IRDs were ~50 to 60% smaller than control larvae. Larvae fed on diet containing IRD-7 displayed ~ 20% and 15% reduction in larval mass and size, respectively (Fig. 3B). Furthermore, larvae fed on inhibitor containing diet showed significant ($p \leqslant 0.05$) reduction in survival rate (Fig. 3C). At day 11, there was ~20% reduction in controlled survival rate (survival rate normalized by control larvae survival rate) of larvae fed on IRD-9 and -12 containing AD as compared to control larvae. PIs fed larvae displays early and sharp decrease in larval survival rates, in case of IRD-7 and -12, it is followed by partial recovery as the feeding period extends. This is might be due to expression of PIs insensitive proteases and overexpression of proteases, which might help insect to overcome the lethal and detrimental effect of inhibitors (Dunse et al., 2010a,b; de Oliveira et al., 2013).

Evaluation of nutritional parameter like Efficiency of Conversion of Ingested Food (ECI), Efficiency of Conversion of Digested Food (ECD) and Approximate Digestibility (Ad) revealed that the ingestion of IRD proteins have deleterious effect on growth and rudimentary metabolism of the insect (Table 1). There was direct correlation in the inhibitory potential and reduction in ECI, ECD and Ad. Assessment of these parameters showed that IRD-9 and -12 negatively affect digestive physiology of insect and thus impedes insect growth and survival (Table 1). Inhibition of serine protease activities also obstructs normal developmental pathways leading to delay in pupation and molting, which was also evident from data (Fig. 3B). Our results indicate that IRD-9 and -12 could serve to develop effective inhibitor molecules against gut proteases from *H. armigera*.

In vivo inhibition of gut proteinases in H. armigera larvae reared on PIs

In comparison with control HGP activity, HGP of larvae fed on IRD-9 and -12 showed ~70% and 80% reduction of activity, respectively. Larvae fed on IRD-7 displays ~40–50% of inhibition of HGP activity as compared to control larvae (Fig. 4A). In case of trypsin-like proteinases activity of HGP from larvae fed on IRD-9 and -12 show ~80–85% reduction as compared to control HGP activity, while it is moderately reduced in HGP of larvae fed on IRD-7.