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The defensive functions of plant inhibitors are not restricted to insect enzyme inhibition

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ABSTRACT

Three plant proteinase inhibitors BbKI (kallikrein inhibitor) and BbCI (cruzipain inhibitor) from *Bauhinia bauhinioides*, and a BrTI (trypsin inhibitor) from *B. rufa*, were examined for other effects in *Callosobruchus maculatus* development; of these only BrTI affected bruchid emergence. BrTI and BbKI share 81% identities in their primary sequences and the major differences between them are the regions comprising the RGD and RGE motifs in BrTI. These sequences were shown to be essential for BrTI insecticidal activity, since a modified BbKI [that is a recombinant form (BbKIm) with some amino acid residues replaced by those found in BrTI sequence] also strongly inhibited insect development. By using synthetic peptides related to the BrTI sequence, YLEAPVARGDGGLA-NH_2 (RGE) and IVYYPDRGETGL-NH_2 (RGE), it was found that the peptide with an RGE sequence was able to block normal development of *C. maculatus* larvae (ED_{50} 0.16% and LD_{50} 0.09%), this being even more effective than the native protein.

1. Introduction

Peptidase inhibitors have been previously documented to have a detrimental effect on insect development and larvae survival. These two parameters, insect development and larvae survival, are well accepted for use in experimental studies of insecticidal activity (Carlini and Grossi-de-Sá, 2002; Macedo et al., 2004).

Cowpeas [*V. unguiculata* (L.) Walp.] are of key importance as dietary staples for millions of people around the world, especially for poorer populations. One of the most important insect pests which infests cowpeas is the bruchid weevil, *Callosobruchus maculatus* (Fabricius) (Coleoptera), which attacks the seeds during storage and severely affects both the quality and yield of the harvest due to low seed germination. During periods of severe infestation, the post-harvest seed losses, caused by *C. maculatus*, can reach up to 100% within a period of 6 months. In order to enhance crop resistance, there are numerous strategies mediated by metabolic products, including primary compounds (e.g. peptidases inhibitors, de Oliveira et al., 2001; Oliveira et al., 2007; Fan and Wu, 2005 and *Bacillus thuringiensis* protein toxin, Promdonkoy and Ellar, 2000); and secondary compounds (e.g. alkaloids and tannins, Dixon and Sumner, 2003).

The use of naturally occurring plant peptidase inhibitors to target insect digestive enzymes has received serious consideration in dealing with insect pests. Numerous bioassays and experiments, where insects are fed with transgenic plants, have shown that

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these proteins can delay insect growth and development, as well as causing insect starvation and death (Hilder et al., 1987; Koiwa et al., 1998; Schuler et al., 1998; Mosolov et al., 2001). Bruchids depend mainly on cysteine peptidases for protein digestion (Silva et al., 2001). Indeed, plant cystatins decrease coleopteran digestive cysteine peptidase activity *in vitro* (Walsh and Strickland, 1993; Koiwa et al., 1998). Cystatin expression in transgenic plants has been shown to be successful in increasing host-plant resistance. Furthermore, rice cystatin expressed in transgenic potatoes inhibited larval growth and caused the death of the Colorado potato beetle (Zhao et al., 1996; Koiwa et al., 1998, 2000). An alternative method that is currently used commercially for controlling certain insect species is the use of *B. thuringiensis* Cry toxins, which are insect specific (Maagd et al., 2003; Walker et al., 2000). More recently, Cry1A was shown to exert its pathological effects on the insect *Manduca sexta* as it binds to two specific receptors, cadherin (Bt-R1) and aminopeptidase-N (APN). To begin with, the monomeric toxin binds to Bt-R1 promoting formation of a pre-pore and inducing some structural changes. Secondly, the pre-pore interacts with APN and is inserted into the target membrane forming pores and leading to cell death (Zhang et al., 2005).

In this study, peptidase inhibitors were isolated from seeds of *Bauhinia*, a leguminous plant of the Fabaceae family and Caesalpinodae subfamily, widespread in tropical and subtropical regions. Inhibitors were isolated and characterized from various species of *Bauhinia* ssp., and investigated on distinct biological models (Sampaio et al., 1996; Oliva et al., 1996, 2000; Nakahata et al., 2006). One of the inhibitors characterized was the trypsin inhibitor BrTI, isolated from *Bauhinia rufa* seeds. This plant Kunitz type inhibitor contains an RGD sequence, and it has been shown that the synthetic peptide YLEPVARGDGGLA-NH_2, which comprises the RGD motif, inhibits B16F10 cell adhesion. However, when Asp9 is replaced by Glu, as in the peptide YLEPVARGEGGLA-NH_2, the cell attachment is not affected (Nakahata et al., 2006).

One view is that the unusual presence of an RGD/RGE sequence in plant proteins suggests that they may be involved in defense strategies against living organisms. In order to evaluate this hypothesis, we investigated the contribution of the sequences comprising the motifs RGD/RGE for the toxic effect of BrTI on *C. maculates* larvae development by replacing them in BbKI, since this inhibitor affects insect survival. In this study, synthetic peptides based on the primary sequence of BrTI were also assessed.

2. Results and discussion

The use of cysteine peptidase inhibitors, cystatins, on coleopteran pests has been attributed to their exogenous peptidase inhibitory activity (Dixon and Sumner, 2003; Oliveira et al., 2007; Zhu-Salzman et al., 2003). Hence, a mixture of the two inhibitors BbKI and BbCI or BrTI was used to observe its effect on *C. maculatus* development.

BbKI inhibits the serine peptidases trypsin, plasma kallikrein and plasmin, and also affects activity of trypsin-like enzymes from digestive tracts of the insects *Abracris flavolineata* (Orthoptera), *Musca domestica* (Diptera), *Periplaneta Americana* (Dictyoptera), *Spodoptera frugiperda* (Lepidoptera), *Diatraea saccharalis* (Lepidoptera) and *Tenebrio molitor* (Coleoptera) (Andrade et al., 2003). BbCI inhibits activity of serine peptidases, human neutrophils elastase, pancreatic elastase and the cysteine peptidase cathepsin L (de Oliveira et al., 2001; Araújo et al., 2005; Oliva and Sampaio, 2008). BrTI has been shown to inhibit trypsin (K_{iapp} 2.9 nM) and human plasma kallikrein (K_{iapp} 14.0 nM), but not other related enzymes (Nakahata et al., 2006). BbI (a mixture of BbCI and BbKI) does not affect *C. maculatus* development, whereas, by contrast BrTI displayed toxic activity with 0.5% (w/w) incorporation in artificial seeds and was lethal at 2.0% (w/w) (Fig. 1, Table 1).

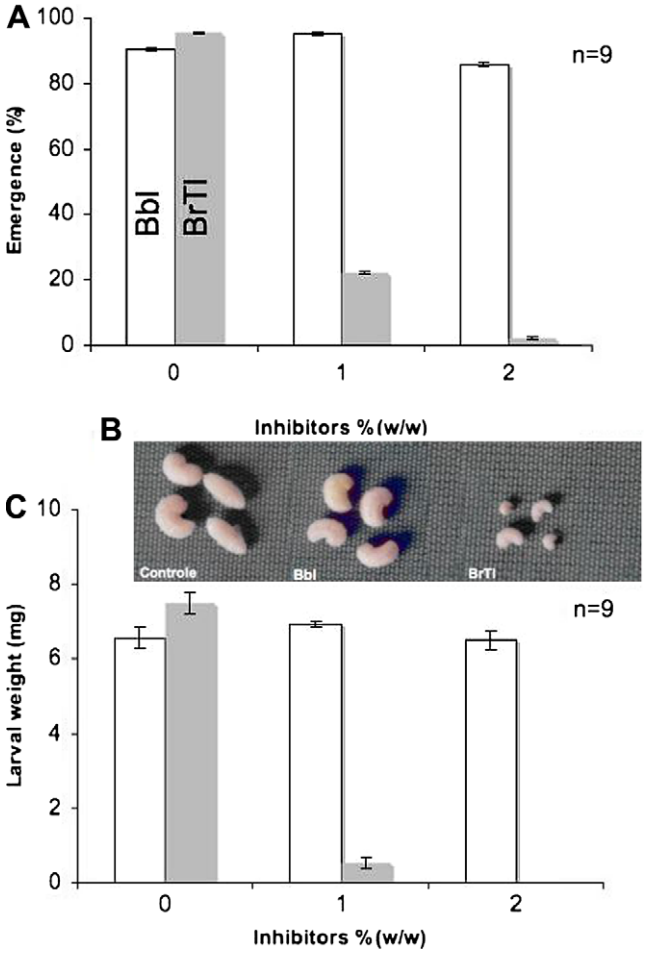

Fig. 1. Effect of BrTI and BbI (homogenous mixture of BbCI and BbKI) on *C. maculatus* larvae developed in artificial seeds. (A) Emergence, (B) photography of *C. maculatus* larvae from the fourth instar developed in artificial seeds incorporated with 1% (w/w) of BbI, BrTI and without (control) in artificial seeds and (C) larval weight.

Table 1
Effect of inhibitors and peptides on *C. maculatus* development.

Inhibitors	Treatment % (w/w)	Larval weight (mg) ± SE	Emergence (%) ± SE
BrTI	0	7.5 ± 0.388	95.5 ± 0.333
	1	0.5333 ± 0.533	22.2 ± 0.333
	2	0	2.2 ± 0.333
BbI	0	6.566 ± 0.296	90.4 ± 0.333
	1	6.933 ± 0.066	95.22 ± 0.333
	2	6.733 ± 0.251	85.7 ± 0.577
BbKIm	0	8.6 ± 1.124	77.8 ± 0.333
	0.9	0.533 ± 0.273	22 ± 0.333
Peptides			
Y-RGD	0	7.666 ± 0.333	77.9 ± 0.333
	0.1	7.233 ± 0.1452	88.9
	0.2	8.166 ± 0.120	88.9 ± 0.333
Y-RGE	0	4.216 ± 0.612	88.9 ± 0.333
	0.1	0.363 ± 0.057	77.9 ± 0.333
	0.2	0.033 ± 0.033	66.6 ± 0.577
I-RGE	0	2.666 ± 0.115	88.9 ± 0.333
	0.1	2.533 ± 0.066	84.4
	0.2	2.000 ± 0.115	66.6 ± 0.333

Mean of determinations from different concentrations of the inhibitors and peptides, each assay in triplicate (standard error). Y-RGD: YLEPVARGDGGLA-NH_2, Y-RGE: YLEPVARGEGGLA-NH_2; I-RGE: IVYYPDRGETGL-NH_2.

Our results indicate that only BrTI affects larval development suggesting that a specific structural feature is involved in insecticidal activity, aside from its inhibitory properties. As these