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Podborer (*Helicoverpa armigera* Hübn.) does not show specific adaptations in gut proteinases to dietary *Cicer arietinum* Kunitz proteinase inhibitor

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Abstract

We investigated the response of *Helicoverpa armigera* larvae towards ingestion of *Cicer arietinum* Kunitz proteinase inhibitor (CaKPI), which caused antagonistic effects on developing *H. armigera* larvae. CaKPI-degrading proteinases were not detectable in either control or sensitized larvae. There were negligible increases in total proteinase activity, as well as in trypsin-like and chymotrypsin-like activities of *H. armigera* gut proteinases (HGPs). Decrease in sensitivity of HGPs to inhibition by CaKPI was not observed when the inhibitor was fed suggesting that the insect had not shown a specific adaptive response to dietary CaKPI. Semi-quantitative reverse transcriptase polymerase chain reaction (Q RT-PCR) analysis showed a general up-regulation of proteases in larvae that ingested CaKPI and a specific regulation of individual transcripts was not observed. CaKPI had maximum inhibitory activity against HGP derived from fourth instar larvae. CaKPI was equally potent in inhibition of HGPs derived from larvae fed on different host plants, as well as various proteinase inhibitors (PIs) to which larval adaptation was previously reported. The lack of larval response to CaKPI was attributable to the atypical active site sequence and inhibitory activity of CaKPI and/or to the pre-adaptation of *H. armigera* larvae due to the constant exposure to basal levels of CaKPI in chickpea seeds or a chickpea seed-based diet.

Keywords: *Cicer arietinum*; *Helicoverpa armigera*; Insect response; Proteinase; Proteinase inhibitor

1. Introduction

Plant-derived proteinase inhibitors (PIs) have received significant attention for developing resistance in crop plants towards the lepidopteran insect pest *Helicoverpa armigera* (Boulter, 1993; Duffey and Stout, 1996; Jouanin et al., 1998; Schuler et al., 1998; Carlini and Grossi-de-sa, 2002; Murdock and Shade, 2002; Ferry et al., 2004). PIs inhibit the digestive larval gut proteinases and impair amino acid intake, thus retarding the growth and development of larvae (Harsulkar et al., 1999; Telang et al., 2003; Srinivasan et al., 2005) and also decreasing the fertility and fecundity of the adult moths (deLeo and Gallerani, 2002; Telang et al., 2003; Tamhane et al., 2005). However, the ability of some phytophagous insects to respond to dietary PIs by a dynamic regulation of digestive proteinases allows them to adapt, and overcome the antimetabolic effects (Patankar et al., 2001). Adaptation of insects to dietary PIs has been observed with a diverse variety of PIs including serine PIs like soybean (*Glycine max*) Kunitz trypsin inhibitor (Bown et al., 1997;

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Mazumdar-Leighton and Broadway, 2001a, b;), tobacco (*Nicotiana tobacum*) PI (Brito et al., 2001), potato (*Solanum tuberosum*) PI-II (Broadway and Duffey, 1986; Jongsma et al., 1995), aprotinin (Gatehouse et al., 1997) as well as cysteine PIs, e.g., oryzacystatin I (Girard et al., 1998a, b) and soycystatin-*N* (Moon et al., 2004).

Among the different types of chickpea (*Cicer arietinum*) seed PIs, the Bowman Birk-type PIs (BBIs) (Belew et al., 1975; Smirnoff et al., 1976) were highly susceptible to degradation by HGPs (Giri et al., 1998), thus their contribution towards insect tolerance is still unresolved. On the other hand, the low-expressing CaKPI (*C. arietinum* Kunitz PI—AY635930) not only inhibited in vitro HGPs activity but also showed significant antimetabolic effects, causing antagonism towards larval growth and development (Srinivasan et al., 2005). The present paper assesses responses towards CaKPI, when fed as a purified recombinant protein to *H. armigera* larvae, to determine whether the insect is able to adapt to CaKPI in a similar manner to its adaptation to other PIs.

2. Materials and methods

2.1. Materials

Bovine trypsin, chymotrypsin and elastase, benzoyl-DL-arginyl-*p*-nitroanilide (BA*p*NA), succinyl-alanyl-alanyl-alanyl-prolyl-leucyl-*p*-nitroanilide (SAAAPL*p*NA), succinyl-alanyl-alanyl-alanyl-*p*-nitroanilide (SAAA*p*-NA), phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-l-phenylalanine chloromethyl ketone (TPCK), *N*-*p*-tosyl-l-lysine chloromethyl ketone (TLCK) and elastatinal were procured from Sigma Chemicals, USA. First strand cDNA synthesis kit and proof-reading DNA polymerase were from Clontech, USA. X-ray films and developer were purchased from Kodak, India. CaKPI was expressed as a recombinant protein in the yeast *Pichia pastoris* and purified as described previously (Srinivasan et al., 2005). All other chemicals used were of analytical grade and procured locally.

2.2. Rearing of insects

Healthy, actively feeding *H. armigera* larvae collected from fields were transferred onto artificial diet and maintained. The composition of the diet was as described by Nagarkatti and Prakash (1974): the components of a 650 mL diet include chickpea seed meal, 77 g; wheat germ, 5.6 g; dried yeast powder, 19.2 g; casein, 12.8 g; ascorbic acid, 4.6 g; methyl para-hydroxy benzoate, 1.5 g; sorbic acid, 0.8 g; streptomycin sulphate, 0.2 g; cholesterol, 0.2 g; formaldehyde, 1 mL; multivitamin drops, 0.8 mL; vitamin E, 12 g, and agar, 10 g. The pupae were then transferred into individual 50 mL screw-capped vials containing about 5 g soil bed. Once adult moths emerged, they were transferred into polyethylene jars covered at the mouth with a black cotton cloth. Adult moths were provided with sterile cotton swabs imbibed with a liquid diet of 10% (w/v) honey or sucrose and 1% (w/v) vitamin E. To ensure greater genetic homogeneity among test populations, the insects were maintained on a control diet for a minimum of three generations, after which they were used for in vivo assays.

2.3. Preparation of fresh HGPs

Five hundred milligrams of tissue was weighed out from freshly dissected *H. armigera* larval midguts. Gut tissue was frozen in liquid nitrogen, ground to a fine powder and extracted in 500 μL of 200 mM Glycine-NaOH buffer (pH = 10.0) for 2 h at 4 °C. The extract was then centrifuged at 12,000 *g* (4 °C, 10 min) and the clear supernatant was used for all solution assays. 5 g *H. armigera* fecal matter was extracted separately in 20 mL of 200 mM Glycine–NaOH buffer (pH = 10.0) for 2 h at 4 °C. To this, 500 μL of gut extract, prepared as described above, was added, the volume made up to 25 mL with 200 mM glycine–NaOH buffer (pH = 10.0) and used for in gel inhibitor activity visualizations.

2.4. Stability of inhibitor towards proteolytic degradation

Five micrograms of CaKPI was mixed and incubated with 5 μg of each, bovine trypsin and chymotrypsin, respectively, and incubated at 37 °C for 0, 30 and 180 min. As a positive control, 5 μg of untreated CaKPI was incubated for 180 min. After incubation, the mixtures were immediately denatured with SDS sample buffer (containing 2-mercaptoethanol) and separated by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), after which the entire gel was stained with Coomassie Brilliant Blue R-250 (CBB R-250). Structural stability of CaKPI towards trypsin and chymotrypsin was determined by the appearance of smaller protein fragments, and a decrease in the staining of the intact protein band compared to the untreated (control) sample. Similarly, 2 μg of CaKPI was mixed and incubated with 10 μL of a fresh HGPs preparation (corresponding to 0.05 units of azocaseinolytic activity, AzU min^{-1}; described in the next section) and incubated at 37 °C for 0, 30 and 180 min. As a positive control, 2 μg of untreated CaKPI was incubated for 180 min. After incubation, the mixtures were immediately separated by 12% native polyacrylamide gel electrophoresis (PAGE) following which the entire gel was equilibrated in 200 mM glycine–NaOH (pH = 10.0) for 5 min at 4 °C, followed by a freshly prepared HGPs solution (as described in previous section) for 10 min at 4 °C and finally rinsed in 200 mM glycine–NaOH (pH 10.0). The gel was overlaid on X-ray film whilst taking care not to introduce any air