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The impact of ingested potato type II inhibitors on the production of the major serine proteases in the gut of *Helicoverpa armigera*

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ABSTRACT

The flowers of the ornamental tobacco produce high levels of a series of 6 kDa serine protease inhibitors (NaPIs) that are effective inhibitors of trypsins and chymotrypsins from lepidopteran species. These inhibitors have a negative impact on the growth and development of lepidopteran larvae and have a potential role in plant protection. Here we investigate the effect of NaPIs on the activity and levels of serine proteases in the gut of *Helicoverpa armigera* larvae and explore the adaptive mechanisms larvae employ to overcome the negative effects of NaPIs in the diet. Polyclonal antibodies were raised against a *Helicoverpa punctigera* trypsin that is a target for NaPIs and two *H. punctigera* chymotrypsins; one that is resistant and one that is susceptible to inhibition by NaPIs. The antibodies were used to optimize procedures for extraction of proteases for immunoblot analysis and to assess the effect of NaPIs on the relative levels of the proteases in the gut and frass. We discovered that consumption of NaPIs did not lead to over-production of trypsins or chymotrypsins but did result in excessive loss of proteases to the frass.

1. Introduction

Plant protease inhibitors (PIs) are part of a natural defense mechanism against herbivores and pathogens (Dunse and Anderson, 2011; Fan and Wu, 2005; Stevens et al., 2012), which has led researchers to examine their potential application in transgenic plants for protection against insect pests. There are many reports of lepidopteran species exhibiting reduced growth, development and survival after ingestion of protease inhibitors that have been incorporated into artificial diets (Bown et al., 2004; Srinivasan et al., 2005) or expressed in transgenic plants (Christeller et al., 2002; Dunse et al., 2010b). However, this response is not obtained with all PIs and no PIs have provided the level of sustainable plant protection required for commercial development (Abdeen et al., 2005).

Several mechanisms have been proposed to explain the detrimental impact of protease inhibitors on larval growth and survival. The most obvious mechanism is formation of a stable complex with digestive proteases which delays or blocks protein digestion, and limits the availability of essential amino acids required for insect growth, development and reproduction (Bown et al., 1997; Broadway, 1995; Gatehouse et al., 1997). Some insect species have been reported to respond to inhibition of gut proteases by hyperproduction of proteases to swamp the ingested PIs and allow digestion to occur (De Leo et al., 1998; Markwick et al., 1998). However, depending on PI levels this can lead to substantial loss of protein to the frass (Broadway, 1995; De Leo et al., 1998; Zhu-Salzman et al., 2003). This in turn limits the bioavailability of essential amino acids for protein synthesis and consequently leads to impairment of growth and development (Abdeen et al., 2005; Broadway, 1995; Markwick et al., 1998). In contrast, some insects are not severely affected by ingestion of PIs. These insects adapt to PIs in their diet by switching to different classes of proteases (Broadway, 1996; Jongsma and Bolter, 1997; Patankar et al., 2001; Winterer and Bergelson, 2001), or by producing inhibitor-insensitive proteases (Dunse et al., 2010b; Volpicella et al., 2003). Another adaptation to PIs is the production of proteases that degrade the PIs in the midgut (Giri et al., 1998; Gruden et al., 1998). Proteolysis of dietary PIs by insect proteases not only reduces the anti-nutritional effect of the PIs on digestive proteases, but also provides an additional source of amino acids (Telang et al., 2005).

It is often difficult to determine which of these adaptive mechanisms are operating in a particular insect pest due to limitations in techniques used to assay gut responses to PIs. Characterizing the effect of PI ingestion on insect digestion has been limited to measuring changes in protease activity or transcription

Abbreviation: NaPI, *Nicotiana alata* proteinase inhibitor.

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of protease genes (Bown et al., 2004; Mazumdar-Leighton and Broadway, 2001). Protease activity is often difficult to measure accurately as activity can be affected by degradation of the proteases during extraction and storage or by the use of inappropriate substrates. Furthermore, protease activity measurements do not detect proteases that are complexed with inhibitors. In this paper we describe the use of enzyme assays and specific antibodies to quantify the levels of both active and inactive trypsins and chymotrypsins, after ingestion of the four trypsin and two chymotrypsin inhibitors produced from the multidomain NaPI precursor (proNaPI).

ProNaPI is a multidomain potato type II protease inhibitor from the ornamental tobacco *Nicotiana alata*. It is composed of six 6 kDa domains; two with chymotrypsin reactive sites (C1, C2) and four with trypsin reactive sites (T1–T4) (Atkinson et al., 1994; Lee et al., 1999). The precursor is processed during transit through the secretory pathway to release six 6 kDa PIs which are stored in the vacuole (Miller et al., 1999). When fed to *Helicoverpa punctigera* or *Helicoverpa armigera* larvae, the 6 kDa PIs were lethal to some larvae, but generally reduced growth and development without mortality (Charity et al., 1999; Dunse et al., 2010b; Heath et al., 1997). We have reported that *H. armigera* larvae that survive consumption of the six 6 kDa inhibitors (NaPIs) have elevated levels of a NaPI-resistant chymotrypsin (Dunse et al., 2010b). Here we describe the use of specific antibodies for the NaPIs as well as *Helicoverpa* chymotrypsins and trypsins to investigate the effect of NaPI consumption on protease production as well as stability of the NaPIs in the midgut of the major agricultural pest, *H. armigera*.

2. Materials and methods

2.1. Production of NaPI affinity column

DNA encoding the chymotrypsin inhibitor (C1) and the trypsin inhibitor (T1) from NaPI was cloned into the pET11a expression vector (Novagen) and expressed in BL21 (DE3) cells as described by Schirra et al., 2001. The purified lyophilized C1-T1 dimer (7.2 mg) was immobilized onto cyanogen bromide-activated Sepharose 4 FastFlow resin (1140 mg, Sigma-Aldrich) according to the manufacturer's instructions.

2.2. Isolation of trypsins from H. punctigera gut

H. punctigera larvae (42) were reared to fourth instar on a haricot bean artificial diet (Teakle et al., 1985) and midguts were excised and homogenized (Sorvall Omni-mixer; 1 min) in ice-cold extraction buffer (41 mL; 10 mM Tris-HCl, pH 8.0; 5 mM EDTA; 10% glycerol; 2% insoluble polyvinylpyrrolidone; 0.01% NaN_3) prior to centrifugation (12,000x *g*; 30 min; 4 °C). The supernatant was incubated with chymostatin (2 μM; 30 min; 4 °C) to inactivate the chymotrypsins, before it was loaded onto an NaPI affinity column that had been equilibrated with ice-cold equilibration buffer (50 mL; 50 mM Tris-HCl, pH 8.5; 20 mM $CaCl_2$). After extensive washing with equilibration buffer the bound proteins were eluted with a linear gradient (31.5 mL) of 0–7 M urea, pH 3.0 over 90 min and elution fractions (350 μL) were neutralized immediately with 50 μL of 1 M Tris-HCl, pH 8.0. Fractions containing protein were assayed for trypsin activity (Dunse et al., 2010b) and the trypsins were then purified further by RP-HPLC using a Brownlee RP300 C8 analytical column (4.6 × 100 mm) and the Beckman BioSys 2000 purification system. Samples were filtered through 0.22 μm disposable membranes (Millipore) before loading onto the column in buffer A (0.1% [v/v] trifluoroacetic acid [TFA]) at 1 mL/min. Proteins were eluted with a linear gradient of 0–100% buffer B (60 mL; 60% [v/v] acetonitrile, 0.089% [v/v] TFA) over 60 min. Elution of proteins was monitored at 215 nm. Proteins were subjected to N-terminal sequencing by Rosemary Condron at the Protein Chemistry Laboratory, La Trobe University using automated Edman degradation and a Hewlett-Packard G1005a protein sequencing system.

2.3. Isolation of cDNAs encoding H. punctigera trypsins

Total RNA was prepared from *H. punctigera* larvae (4th instar) using Trizol reagent (Invitrogen Life Technologies). Two partial cDNAs were obtained using the Superscript cDNA synthesis kit (Invitrogen Life Technologies) and PCR with oligonucleotides complementary to conserved peptide motifs within trypsin sequences. The first primer was designed based on the serine protease motif, TAAHC (TrF1-ACC GCT GCT CAC TGC AC) and the second primer was designed to the trypsin specific motif, DQCQG (TrR1-GAC CAG TGC CAG GGT GAC) that are both present in the active site of trypsins. The partial cDNAs were used to isolate full length clones from the *H. punctigera* midgut cDNA library as described in Dunse et al. (2010b).

2.4. Bacterial expression of HpTry1A

DNA encoding the zymogen for the major digestive trypsin from *H. punctigera* (proHpTry1A) (lacking the N-terminal endoplasmic reticulum signal sequence) was cloned into the pQE30 plasmid (Qiagen) using *Bam*HI and *Hin*dIII restriction sites. Protein was expressed in *Escherichia coli* strain M15 and purified as described in the QIAexpressionist manual (Qiagen). Cells were harvested 4 h after induction and the insoluble expressed histidine tagged protein (6H.proHpTry1A) was purified from the cell pellet under denaturing conditions using TALON® metal affinity resin (Clontech). Bound protein was eluted from the resin with 8 M urea, 100 mM sodium phosphate, 10 mM Tris-HCl, pH 4.5. The eluted protein was concentrated using an ultrafiltration membrane (5 kDa cut-off; Millipore) in an Amicon stirred cell at 4 °C and was stored in 1 M urea, 100 mM sodium phosphate, 10 mM Tris-HCl pH 8.0 at −20 °C.

2.5. Antibody production

The recombinant trypsin, 6H.proHpTry1A (100 μg in 1 mL of phosphate buffered saline [PBS]) was mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich) and injected subcutaneously into a rabbit (New Zealand White). Booster immunizations were administered every four weeks and consisted of protein (100 μg in 1 mL of PBS) mixed with 1 mL of Freund's incomplete adjuvant (Sigma-Aldrich). Pre-immune serum was collected before injection and immune serum was collected 12–14 d after every booster injection (x5). The same procedure was used to raise antibodies against the recombinant *H. punctigera* chymotrypsins, 6H.proHpCh2A and 6H.proHpCh5 (Dunse et al., 2010a).

The six 6 kDa proteinase inhibitors (1.2 mg) derived from the 42 kDa NaPI precursor were purified from the stigmas of mature *N. alata* flowers as described in Heath et al. (1997). The anti-NaPI antibody was raised against the purified 6 kDa inhibitors and then Protein A purified as described by Atkinson et al. (1993).

2.6. Insect bioassays

H. punctigera larvae were from a laboratory colony generated from larvae collected from the field in Victoria. *H armigera* larvae were raised from eggs supplied by CSIRO Ecosystem Sciences, Canberra. *H. armigera* feeding trials were conducted as described by