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Effects of cysteine protease inhibitors on oviposition rate of the western flower thrips, *Frankliniella occidentalis*

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Abstract

Proteolytic activity in whole insect extracts of the western flower thrips, *Frankliniella occidentalis*, was found to belong predominantly to the class of cysteine proteases. The pH optimum of the general proteolytic activity was determined to be 3.5, which is low when compared to other insects using cysteine proteases for protein digestion. The proteinaceous cysteine protease inhibitors chicken cystatin, potato cystatin and sea anemone equistatin inhibited in vitro more than 90% of the protease activity. To test in vivo the biological effect of such inhibitors on the oviposition rate of western flower thrips, recombinant potato cystatin and equistatin were fed to adult females. A gradual reduction in oviposition rate to about 45% of control was observed when reared on these PIs for a period of 5 days, with no increase in mortality. These results are discussed in the light of the application of protease inhibitors in transgenic plants to control this insect pest.

1. Introduction

The Western flower thrips (WFT), *Frankliniella occidentalis* (Thysanoptera) is currently the most prevalent insect pest in greenhouses on vegetable and ornamental crops in the Netherlands, and has been so since 1983 (Mantel and De Vrie (1988). This insect is highly polyphagous, having been reported to live on at least 240 species of 62 different families of plants (Loomans et al., 1995). Economic damage is not limited to greenhouses as WFT also causes yield losses in field crops such as onion, cotton, chilli, and capsicum; in fruit crops such as citrus, apple, and strawberry; in plantation crops like tea and in many more. The small size and the secretive (thigmotactic) habit of hiding in the inner whorls of flowers and buds makes chemical control of WFT difficult. WFT incidence results in direct damage of pierced and emptied cells resulting in loss of photosynthesis and stunted growth (Harrewijn et al., 1996), and in indirect damage by tospovirus transmission (Wijkamp et al., 1995). Several protocols exist for testing WFT resistance on plants or plant tissues, e.g. measurement of (silver) damage, population dynamics (van Dijken et al., 1995), reproduction (Soria and Mollema, 1995) and egg production (Brouwer et al., 1996). Oviposition rate is a suitable parameter to assess the effects on WFT of metabolic inhibitors like protease inhibitors (PIs), because the production of eggs relies heavily on the conversion of plant protein into yolk protein. A precedent is Wolfson and Murdock's (1990) demonstration that sublethal levels of E64 can have remarkable effects on the fecundity of Mexican bean beetle.

Proteases are comprised of four dominant classes (cysteine, serine, aspartic and metallo proteases) based on the amino acid or metal ion involved in the catalytic hydrolysis of peptide bonds. The classes of proteases found in the alimentary tracts of insects varies strongly between species (Wolfson and Murdock, 1990). Proteases are usually inhibited by proteinaceous PIs through tight reversible binding to the substrate binding site itself or to subsites, in both cases causing steric hindrance of the enzymatic conversion of normal peptide substrates. PIs are named after the protease they inhibit, and, hence, there are serine PIs, cysteine PIs and so on. Effective

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inhibition of proteolysis may require multiple PIs to act on all major gut proteases. Ingestion of effective PIs may result in stunted growth, lowered oviposition rate and increased mortality of insects due to lowered release of amino acids from food protein (Jongsma and Bolter, 1997). However, insects may also overcome the effect of PIs by proteolytic inactivation of PIs (Michaud, 1997; Girard et al., 1998b) or by the induction of an inhibitor-insensitive complement of proteases (Bolter and Jongsma, 1995; Jongsma and Bolter, 1997).

Our interest was in identifying PIs antagonistic to WFT. The characterisation of the dominant gut proteases and the pH optima for their activity allows the selection of effective PIs against the gut proteases. This study describes the partial characterisation of WFT proteolytic activity and identification of PIs active in vitro. Specific cysteine PIs are subsequently tested in vivo against adult female WFT to observe effects on oviposition rate. The response of WFT to ingestion of PIs and the potential of expressing cysteine PIs in plants to manage this major insect pest is discussed.

2. Materials and methods

2.1. Materials

Unless otherwise noted all chemicals were purchased from Sigma Chemical Co. (St Louis, MO). The following abbreviations are used: $CdCl_2$, Cadmium chloride; E-64, trans-epoxy succinyl-L-leucylamido-(4-guanidino)-butane; PI, protease inhibitor; PI1(Calbiochem), potato proteinase inhibitor 1; PI2 potato proteinase inhibitor 2; PMSF, phenylmethylsulfonyl fluoride; SBBI, soybean Browman Birk inhibitor; STI, soybean trypsin inhibitor; PC, potato cystatin; PCI, potato carboxypeptidase inhibitor; EI, Equistatin; WFT, western flower thrips; FITC, fluorescein isothiocyanate; TCA, trichloracetic acid.

2.1.1. Characterisation of WFT proteases

Adult WFT feeding on chrysanthemum (*Dendranthema grandiflora*) cv. Sunny Casa were collected from the rearing chamber. The small size of WFT did not permit the separation of the guts from the carcass. Hence, whole insects were homogenised in a plastic potter fitting an Eppendorf tube containing 300 μl water. Insoluble debris was removed by centrifugation. The pH optimum for the proteolytic activity in the total WFT extract was determined exactly as described by Jongsma et al. (1996) using [^{14}C] methemoglobin (NEN, DuPont de Nemours). For inhibition assays, 200 μl of this extract was diluted with 800 μl of 200 mM β-alanine-HCL pH 3.5 buffer. The extract was tested against the inhibitors as described by Jongsma et al. (1996).

A second series of inhibition assays was carried out using FITC-labelled methemoglobin with total WFT extract as described by Twining (1984). Potato cystatin (PC, Annadana et al., submitted), equistatin (EI, Rogelj et al. (2000) and PI2 (Beekwilder et al., 2000) were recombinantly produced while chicken cystatin (Zerovnik et al., 1990) was purified from its native source.

The pH optima of extracts of thrips reared on pollen and extracts of pollen alone were determined to demonstrate that the observed inhibition profiles related to thrips derived proteolytic activity. Samples were homogenized separately using a glass potter in a ratio of 1:4 (w/v) of sample with distilled water. Insoluble debris were removed by centrifugation. For the assay the following pH-buffers (200 mM Glycine-HCl pH 3.5; 100 mM Na-acetate pH 4.1; 50 mM MES pH 4.5; 50 mM MES pH 6.5; 50 mM MES pH 7; 100 mM Tris–HCl pH 8.5) were prepared. Thrips or pollen extracts (5 μl each) were mixed into 85 μl of the different pH buffers and 10 μl FITC albumin (1 mg/ml stock) was added. The reactions were allowed to proceed for 1 h at 37 °C, and were terminated by adding 30 μl of 8% TCA on ice. The samples were subsequently centrifuged for 5 min at 1400 rpm and 4 °C. The supernatant (20 μl) was mixed with 180 μl 100 mM Tris–HCl pH 8.5 and the fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.1.2. Bioassay for testing oviposition rate in WFT

Bioassay cages were prepared from transparent perspex tubes 3 cm in length and 3.5 cm diameter. The bottom of the tube was closed with nylon gauze of 120 μm mesh size. The top was sealed with two sheets of stretched parafilm, with a liquid water sandwich of 300 μl in between. The effect of the pure proteins (PIs), was tested in a bioassay using these bioassay cages by using protein solutions in the liquid sandwich. The experiment was conducted with six replications for all PI treatments and with nine replications for the control. Cages with 20 adult female WFT were incubated in climate controlled chambers maintained at 25 °C temperature, 60% relative humidity and 16 h of light. Pollen of mixed plant origins (health product from grocery store), compacted into grains of 1–2 mm diameter, were used as a food source for WFT in our bioassay. Moisture is critical to rehydrate the otherwise dry pollen grains. Only rehydrated pollen has the correct physical consistency for WFT feeding. Hence, bioassay cages were placed gauze side down in a tray, on top of holes of 1 cm^2, permitting the diffusion of moisture. A piece of parafilm with 4 pollen grains on top was placed under the gauze side of the cage (next to the hole). In this way pollen was directly accessible to the WFT through the gauze and easily replaced without disturbing the insects. PIs were diluted into diluted FPLC buffer (1.5 mM Na-citrate 36 mM NaCl, pH 5), which also served as the control solution. All eggs were

deposited inside the PI/FPLC buffer sandwich. Eggs were counted and adult mortality was scored daily. Subsequently, the sandwich and pollen were refreshed daily after CO_2 anaesthesia of the insects.

3. Results

3.1. Characterisation of WFT proteases

A pH profile of protease activity was determined using a range of pH 2 to 12. Protease activity in extracts of total adult WFT was found to be maximal at pH 3.5 with low activity levels at pH values greater than 5 (Fig. 1a). In order to control for the effect of plant proteases present in the ingested pollen a pH profile of pollen protease activity was determined. In contrast to the above results the protease activity of pollen was found to be maximal at pH 8.5 with low values at pH 3.5 suggesting that pollen proteases do not contribute significantly to the activity measured in thrips extracts at pH 3.5 (Fig. 1b). Proteases of adult females when analysed separately from the mixture of males and females had a similar pH optimum (data not shown). At pH 3.5 the protease activity was characterised with non-proteinaceous inhibitors of cysteine, serine, metallo and aspartic protease inhibitors respectively (Table 1). Greater than 80% inhibition was observed with E-64, chymostatin and leupeptin, but less than 10% inhibition was found with benzamidine, PI1, SBBI and STI, suggesting a predominance of cysteine proteases in total WFT extracts. Elastatinal and potato carboxypeptidase inhibitor, a serine and a metallo protease inhibitor respectively, showed 20% inhibition of proteolytic activity.

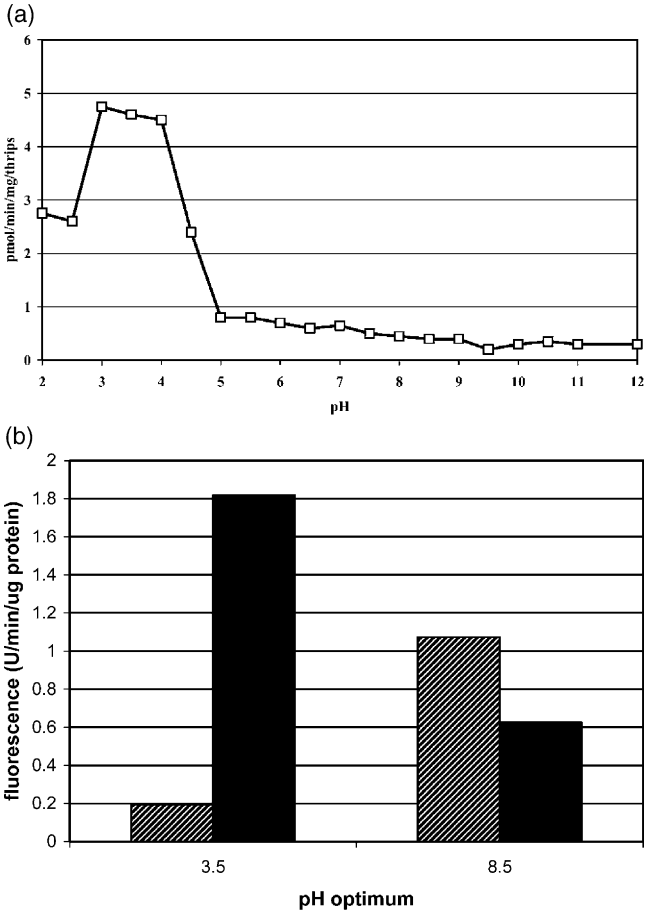

Fig. 1. (a) pH profile of the proteolytic activity of whole WFT extract assayed using [^{14}C]methemoglobin as a substrate. (b) Proteolytic activity of pollen extract (grey bar) and whole WFT extract (black bar) at the pH optima of WFT and pollen extracts respectively (pH 3.5 and 8.5) FITC-labelled albumin as a substrate.

Four proteinaceous PIs and one chemical inhibitor (E64) were subsequently tested for their inhibition of the total WFT proteolytic activity, using FITC-labeled methemoglobin in a fluorescence assay. The cysteine PIs chicken cystatin, E-64, PC and EI showed inhibition in the range of 90–96% whereas PI2, a serine protease inhibitor of trypsin, chymotrypsin and elastase showed 20% inhibition of total WFT proteolytic activity (Table 1).

3.2. Bioassay to test effect of recombinant PC and EI on oviposition rate

In total, more than six bioassays were conducted to study the effect of PC and EI on the oviposition rate of WFT. The results of one representative bioassay are presented. The bioassay was performed in three phases, the first three days of adaptation on control diet (phase I), followed by five days of exposure to PIs (phase II) and the final three days to recover from exposure to PIs by returning to control diet (phase III). The average number of eggs laid in the control on day 1 and 0 of phase I was 2.50 ± 0.18 eggs/female. The average number of eggs by each bioassay cage during day 1 and 0 of phase I was taken as 100% against which egg counts of phase II and III were compared for all treatments. During phase I, when all cages were on control diet, there was no significant change between the cages in oviposition rate on day 1 and 0, indicating good adaptation of the WFT to the conditions of the bioassay.

Two recombinant PIs, PC and EI were used in phase II of the bioassay at 30 μM concentration (PC=0.03% (w/v), and EI=0.06% (w/v)) to assess the effects of PI ingestion on oviposition rate of WFT. On day 1 a drastic reduction in oviposition rate, was observed for all three PI treatments (Fig. 2). Reductions of 38% (PC), 28% (EI) and 45% (PC+EI) in oviposition rate occurred. All treatments recovered on the second day of phase II resulting in 27.5 %, 17 % and 21 % of reduction in oviposition rates respectively (Fig. 2). During the remaining three days a steady downward trend was observed. On day 5, the last day of phase II, PC, EI and PC+EI had oviposition rates of 51, 66 and 52% relative to their orig-

Table 1
Effect of protease inhibitors on hemoglobin hydrolysis by proteases in whole insect homogenates of *Frankliniella occidentalis* adults[a]

Inhibited class of protease	Inhibitor*	Concentration(μM)	Relative activity(% control)	
			[^{14}C]met-hemoglobin	FITC- hemoglobin
Serine proteases	Aprotinin	5	89	
	Benzamidine	1	92	
	Elastatinal	5	80	
	PMSF	5000	87	
	PI1	6	93	
	SBBI	12	95	
	STI	2.5	90	
	P12	5		80
Serine/cysteine proteases	Antipain	100	29	
	Chymostatin	100	13	
	Leupeptin	1000	29	
Cysteine proteases	E-64	10	13	7
	PC	5		5
	Chicken systatin	5		10
Cysteine/aspartic proteases	EI, equistatin	2.5		5
Aspartic proteases	Pepstatin	10	84	
Metallo proteases	PCI	5	80	
	$CdCl_2$	5000	89	

[a] See Methods for abbreviations

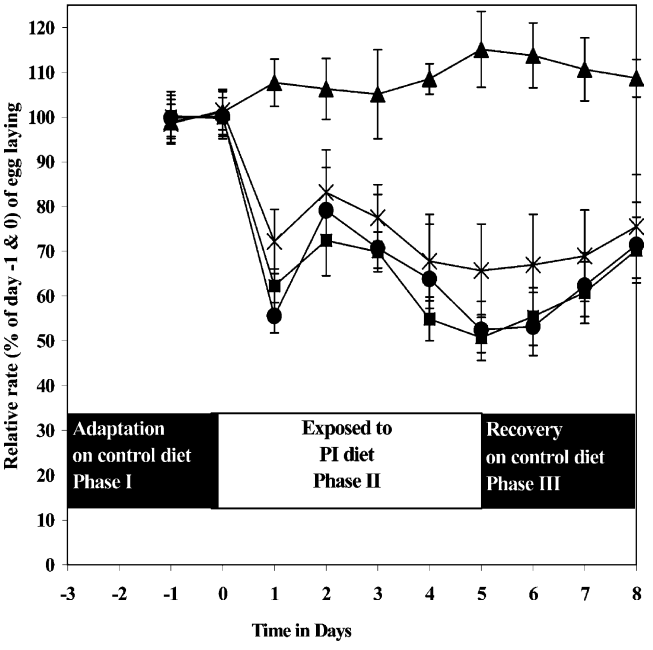

Fig. 2. Influence of dietary PIs on oviposition rate rates of adult WFT. The graph indicates the oviposition rates of WFT reared on pollen grains as food source exposed to 30 μM potato cystatin PC (■), 30 μM equistatin EI (×), and a combination of both PC+EI at 30 μM each (●) vs. a control CON (▲). The points of the graph are generated based on average of six values for the treatments and an average of nine values for CON. All values are represented as a percent of the average number of eggs laid on day 1 and 0 with ±SE bars. The three phases viz. adaptation, exposure to PIs and recovery are indicated on the graph with phase I and III shaded.

inal day 0 rates. Relative to the control on day 5, oviposition rates were 44, 57 and 45% respectively (Fig. 2, Table 2).

In phase III (days 6–8) the insects were returned to the control diet of pollen and buffer. The oviposition rate was found to increase at a similar rate to that at which the decrease had been observed in phase II (Fig. 2).

The relative egg count at the end of phase I, II and III are presented in Table 2. The statistical treatment indicates a significant reduction in oviposition rate compared to control at the end of phase II ($P < 0.001$) and phase III ($P < 0.01$). PC appeared to be more effective than EI and there was no synergistic effect of the combination of PC and EI at 30 μM.

4. Discussion

The objective of this investigation was to evaluate the potential of PIs to control WFT. To this end, the predominant group of proteases in whole insect homogenates was determined, under the assumption that in such extracts the gut proteases will dominate. However, due to the fact that whole insect homogenates were used it cannot be excluded that inhibitors present in the hemolymph create a bias in the remaining activity which was assayed in this report. The dominant group of proteases were identified to belong to the class of cysteine proteases with an activity optimum of pH 3.5. The protease activity with low pH optimum found in whole insect homogenates is not an artefact due to proteases present

Table 2
The effect of PIs on oviposition rates in WFT

Treatment	Concentration	Day 0 (%)[a]	Day 5 (%)[a]	Day 8 (%)[a]	Day 8 (% survival)[b]
-	–	101^{1} (100)	115^{1} (100)	109^{1} (100)	94^{1}
PC	30 μM	99^{1}(98)	51^{2} (44)	70^{2}(64)	96^{1}
EI	30 μM	101^{1} (100)	66^{2} (57)	76^{2} (70)	97^{1}
PC + EI	30+30 μM	100^{1} (99)	52^{2} (45)	71^{2} (65)	97^{1}

[a] The relative number of eggs for days 0 (end of phase I, adaptation to control diet), 5 (end of phase II, exposure to PI diet) and 8 (end of phase III, recovery on control diet) are presented as a percentage of day 1 and 0 for PI treatments with six replications and control with nine replications. Between brackets is the percentage relative to the control of that same day (corrects for interday variation). The least significant differences for days 0, 5 and 8 are 17, 33 and 28% respectively. The treatments are grouped based on significance with values denoted with different superscript numbers differing significantly.

[b] The survival is presented as a percentage of the number alive on day 1.

in dietary pollen. This possibility is excluded by the finding that pollen proteases have a basic optimum at pH 8.5 and activity at pH 3.5 is low. The pH optimum of 3.5 shown by WFT enzymes is low, when compared to those of gut cysteine proteases of other insects like *Psylloides chrysocephala* with a pH optimum of 6–6.5 (Girard et al., 1998a), and *Phaedon cochleariae* and *Ceutorhynchus assimilis* with a pH optimum of 5 (Girard et al., 1998b). Along with cysteine proteases as a dominant class of proteases, some insects like *Leptinotarsa decemlineata* (Wolfson and Murdock, 1987; Gruden et al., 1998) and *Diabrotica undecimpunctata* (Edmonds et al., 1996) in addition have aspartic proteases. In WFT the aspartic protease inhibitor pepstatin showed 16% inhibition of proteolytic activity in total extract. This suggests that in WFT the contribution of aspartic proteases to total gut proteolytic activity may be limited despite the low pH optimum. The inhibition of WFT proteases by elastase inhibitors like elastatinal and PI2 is unexpected, because at pH 3.5 the serine proteases would be expected to be inactive, while at alkaline pH no secondary peak of activity is observed.

WFT were subsequently fed with proteinaceous PIs each at a concentration of 30 μM, which is in the range of concentrations that can be found in plant leaves. Exposure to PC and PC+EI for a limited period of five days resulted in a 55% reduction in oviposition rate relative to the control. A reduction in oviposition rate by more than 50% can have important effects on the exponentially growing population of WFT. Using a simulation model, Yano et al. (1989) predicted a 75% reduction in a whitefly population on tomato, as a result of a 50% reduction in oviposition rate, over an 80-day period. Similarly, Wolfson and Murdock (1990) showed using models that such non-lethal effects could have dramatic effects on the development of economic populations of a pest. A similar simulation model taking into account the relevant life history parameters of WFT on chrysanthemum in greenhouses (18 day egg–egg, 3 eggs/female) predicts a 92% reduction in the density of the population relative to control, at the end of a ninety day period, as a consequence of a 50% reduction in oviposition rate.

Kirk (1985) reported that egg laying in thrips species in general comes to a complete stop within 2–3 days after being deprived of adequate nutrition. In our case we presume that adding proteinase inhibitors blocks the production of free amino acids from food protein, leading to an increasing reduction of the oviposition rate by more than 50% after 5 days. The relatively slow effect of PIs on oviposition rate suggests that there was still a supply of amino acids from dietary or storage protein. However, the steady decrease suggests that this activity can only support a relatively low oviposition rate. In addition, the slow rate of recovery in oviposition during phase III suggests that affected insects need to restore their storage protein reserves before they can produce eggs at the original rate.

Thus, we conclude that cysteine PIs strongly affect oviposition rates, and that additional inhibitors like elastase or carboxypeptidase inhibitors may be required to inhibit the gut activity even more effectively.

The only earlier study on effect of PIs on WFT was conducted by Thomas et al. (1994), who reported that the expression of a serpin elastase inhibitor from *Manduca sexta*, in transgenic alfalfa resulted in delayed WFT damage. However, there was no characterisation of WFT proteases or the effects of PIs on in vitro protease activity and adult oviposition rate. Similar to our results with PC and EI on WFT, Spates and Harris (1984) also showed that reduction in oviposition rate in *Stomoxys calcitrans* occurred upon feeding with PIs. In this case, soybean trypsin inhibitor at 45 μM concentration (0.1% (w/v)) reduced oviposition rate by over 70 %. Interestingly, these authors also observed reduced egg hatch, which is functionally equivalent to a further lowered production of eggs. The larger part of the literature on the effects of PIs on insects concentrates on the development of larvae which unlike adults, require protein for their growth and development. PIs have been reported to cause delay in larval development, stunted growth and to increase larval mortality (Jongsma and Bolter, 1997;

Orr et al., 1994; Ortego et al., 1998). A stronger effect of PC and EI on WFT can be expected if these PIs also affect egg hatch, larval growth and development in addition to their effects on oviposition rate, but these parameters were not investigated in the present study. Future investigations of these parameters are best carried out using transgenic plants as this will simplify experimental procedures, and allow the establishment of long term effects of protease inhibitors on WFT populations.

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