Potential effects of plant protease inhibitors, oryzacystatin I and soybean Bowman-Birk inhibitor, on the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera, Braconidae)

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**Abstract**

Protease inhibitors (PIs) have been shown to cause lethal and sublethal effects on aphids depending on the kind of PI and aphid species. Therefore, these proteins might affect aphid parasitoids directly by inhibiting their digestive proteolysis or indirectly via their development in a less suitable host. In our study, the risk of exposure and the potential effects of soybean Bowman-Birk inhibitor (SbBBI) and oryzacystatin I (OCI) on the aphid *Aphidius ervi* were investigated using artificial diet to deliver PIs. Immunoassays showed that both SbBBI and OCI were detected in the honeydew of aphids reared on artificial diet containing these recombinant proteins at 100 \(\mu\)g/mL. However, only SbBBI was detected in parasitoid larvae, while this PI could not be detected in adult parasitoids emerged from PI-intoxicated aphids. Enzymatic inhibition assays showed that digestive proteolytic activity of larvae and adults of *A. ervi* predominantly relies on serine proteases and especially on chymotrypsin-like activity.

Bioassays using SbBBI and OCI on artificial diet were performed. *A. ervi* that developed on intoxicated aphids had impaired fitness. Thus development and parasitism success of parasitoids exposed to OCI were severely affected. On the contrary, SbBBI only altered significantly female size and sex ratio. Direct exposure to PIs through adult food intake did not affect female’s longevity, while SbBBI and OCI (100 \(\mu\)g/mL) induced 69% and 30% inhibition of digestive protease activity, respectively. These studies made it possible to estimate the risk of exposure to plant PIs and the sensitivity of the aphid parasitoid *A. ervi* to these entomotoxins, by combining immunological, biochemical and biological approaches. First it pointed out that only immature stages are affected by PIs. Secondly, it documented two different modes of effect, according to the nature of the PIs and both host and parasitoid susceptibility. OCI prevented the development of *A. ervi* mainly due to the host susceptibility, whereas SbBBI only induced sublethal effects on the parasitoid, possibly due to both direct action on the parasitoid susceptible proteases, and host-mediated action through size reduction.

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**Keywords:** Aphid parasitoid; Protease inhibitors; Tritrophic interactions; Risk assessment; Transgenic plants

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1. **Introduction**

Protease inhibitors (PIs) from plants exhibiting insecticide effects emerged as an interesting strategy for insect pests control (Jouanin et al., 1998; Carlini and Grossi-de-Sá, 2002; Lawrence and Kouandé, 2002; Ranjekar et al., 2003). Several serine and cysteine PIs...
have been expressed in transgenic plants belonging to different families to enhance their resistance against Lepidoptera (Hilder et al., 1987; De Leo et al., 2001; Falco and Silva-Filho, 2003) and Coleoptera (Lecardonnel et al., 1999; Alfonso-Rubí et al., 2003). The insecticidal effects of PIs against sap-sucking phytophagous insects was established recently (Annadana et al., 2002; Ceci et al., 2003; Rahbé et al., 2003a; Rahbé et al., 2003b).

Aphicidal effects of PIs, especially of some serine and cysteine PIs, have been established by diet incorporation assays. The pea and soybean trypsin–chymotrypsin inhibitors (PsTI-2, SbBBI) belonging to the Bowman-Birk family (Rahbé et al., 2003b) and the mustard-type trypsin–chymotrypsin variant Chy8 (Ceci et al., 2003) induced significant mortality and growth inhibition of the pea aphid *Acrystosiphon pisum*. In addition, SbBBI had deleterious effect on the life parameters of the adult potato aphid *Macrosiphum euphorbiae* (Azouz et al., 2005). The phytocystatin oryzacystatin I (OCI) isolated from rice seeds (Abe et al., 1987) significantly reduced the nymphal survival of *M. euphorbiae* and prevented aphids from reproducing (Azouz et al., 2005). In addition, Rahbé et al. (2003a) showed that OCI significantly reduced adult weight and fecundity of the aphid *Myzus persicae*. Genes encoding PIs are therefore regarded as candidates for expression in transgenic crops and appear a promising strategy to confer resistance against homopteran pests and especially aphids (Deraison, 2002; Ceci et al., 2003; Rahbé et al., 2003b; Azouz et al., 2005). Except for Bt endotoxin expressing plants (Rice and Pilcher, 1998; Kumar and Kumar, 2004), target insects are not usually completely controlled by transgenic plants (Stoger et al., 1999). Consequently aphid limitation by sublethal effects of plant expressing PIs could be complemented by the use of beneficial insects (van Emden, 1999). Thus, it is important to investigate the potential negative side effects of this new technology on non-target insects such as predators and parasitoids.

Several studies investigated the effects of PIs on insect predators (Ashouri et al., 1998; Overney et al., 1998; Walker et al., 1998; Bouchard et al., 2003; Ferry et al., 2003) and parasitoids (Down et al., 1999; Ashouri et al., 2001; Bell et al., 2001; Schuler et al., 2001; Cowgill et al., 2004). They reported that the major digestive proteolytic activity of adult and larvae ladybirds *Adalia bipunctata* (Walker et al., 1998) and *Harmonia axyridis* (Ferry et al., 2003), and the stinkbug *Perillus bioculatus* (Overney et al., 1998) was cysteine protease based. It is only known that OCI reduced the fitness of *P. bioculatus* (Ashouri et al., 1998) but did not affect *H. axyridis* (Ferry et al., 2003). Regarding parasitoids, only the digestive protease activities in the larval stage of ectoparasitoid *Eulophus pennisicornis* (Down et al., 1999) and the larval and adult stage of endoparasitoid *Aphelinus abdominalis* (Azouz et al., 2005) were reported. No deleterious effect of OCI was found in the aphid parasitoids *Aphidius* spp. (Ashouri et al., 2001; Cowgill et al., 2004) and *Diaeretiella rapae* (Schuler et al., 2001) parasitising aphids reared on OCI-expressing plants. However, in laboratory bioassay using *M. euphorbiae* reared on artificial diets supplemented with OCI and SbBBI, we demonstrated that development and parasitism success of *A. abdominalis* were adversely affected (Azouz et al., 2005).

The objective of this study was to characterise and analyse the effects of oryzacystatin I (OCI) and soybean Bowman-Birk inhibitor (SbBBI) on the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) in controlled exposure conditions. *A. ervi* is a solitary endoparasitoid commercialised to control several aphid species in greenhouses. Risks of exposure of *A. ervi* to OCI and SbBBI were investigated first. Secondly, the digestive protease activities of larval and adult stages were determined. Finally, effects of PIs enriched artificial diets were investigated on parasitoid development and parasitism success. Effect of PIs on *A. ervi* adult survival was also studied.

2. Materials and methods

2.1. Reagents

Trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64) and soybean trypsin–chymotrypsin Bowman-Birk Inhibitor (SbBBI) (>95% of purity) were obtained from Sigma. Pepstatin A and chymostatin were supplied by Alexis biochemicals. Ethylenediamine Tetra-Acetic Acid (EDTA) was obtained from Prolabo and pefablock from Interchim. The EnzChekR Protease Assay Kit (fluorescent protein BODIPYR-FL casein) was purchased from Molecular Probes. Oryzacystatin I (OCI) (>95% of purity) was obtained as previously described by Leplé et al. (1995). The polyclonal anti-OCI (Leplé et al., 1995) or anti-SbBBI (Bonadé-Bottino, unpublished results) were raised in rabbits and used as primary antibodies. The secondary antibody peroxidase-coupled goat anti-rabbit IgG (A 0545) was purchased from Sigma. ECL kit and nitrocellulose membrane HybondTM–ECLTM were obtained from Amersham Biosciences.

2.2. Artificial diets

As a control and a basis for the protein dilution, a standard diet adapted for *M. euphorbiae* was prepared as described by Febvay et al. (1988) and modified by Down et al. (1996). OCI or SbBBI were incorporated to the standard diet to obtain the following diets: OCI-100, OCI-250 contained 100 and 250 μg/mL of OCI, respectively; SbBBI-100 and SbBBI-500, contained 100 and...
500 μg/mL of SbBBI, respectively. The diet sachets were prepared as described in Couty et al. (2001b) under aseptic conditions, sterilised by filtration through 0.45 μm filters (Millipore Corp., Bedford, MA) then kept frozen at −20 °C until used.

To expose adult parasitoids to PIs through food intake, sugar solution of 70% (w/v) 1:1 glucose–fructose mixture was used as a sugar source for A. ervi females. Twenty μg/mL of Bovine Serum Albumin (BSA) was added to this sugar solution to stimulate the protease activity of this parasitoid. This control solution was then supplemented with BSA, OCI or SbBBI at 100 μg/mL (BSA100, OCI100 and SbBBI100, respectively).

2.3. Insects

The LB05 Sm clone of the potato aphid M. euphorbiae provided by Yvan Rahbé (INRA-INSA Villeurbanne, France) was reared in the laboratory on potato plants (Solanum tuberosum, cv Désirée) in a controlled environment room (T = 20 ± 1 °C; RH = 60 ± 5%; 16L/8D). Neonate nymphs (12 h old) used in the experiments were issued from females synchronised on an excised potato leaf isolated in a plastic box.

Mummies of A. ervi purchased from Biobest (Belgium) were placed in plastic boxes (17 × 11 × 7 cm) until adult emergence under the same conditions as described above. Emerging male and female parasitoids were kept together and fed ad libitum on cottonwool imbibed with sugar solution (glucose and fructose 70% (w/v)) during 48 h before infesting aphids.

2.4. Insect bioassays

2.4.1. Effects of protease inhibitors on M. euphorbiae size

In order to assess the effect of PIs on M. euphorbiae size, neonate aphids were fed for four days on OCI-100, SbBBI-100 and control diets. Thirty-five-days-old nymphs were individually measured from the tip of the head to the base of the cauda (length) and across the metathorax (width), under a microscope using an eyepiece graticule. Experiment was conducted in a controlled environment room (T = 20 ± 1 °C; RH = 60 ± 5%; 16L/8D).

The expressed level of PIs in plant foliage is from 0.05% to 2.5% of total soluble proteins (Leplé et al., 1995; De Leo et al., 1998; Charity et al., 1999; Lee et al., 1999). The level of total soluble proteins in the phloem is less than 5 mg/mL in non-cucurbit species (Fisher et al., 1992; van Helden et al., 1994; Ishiwatari et al., 1995; Schobert et al., 1998; Foissac et al., 2002). According to Rahbé et al. (2003a) the amounts expressed into leaf tissue seems to be close to the amount present in the phloem sap. Therefore we chose to deliver to aphids a PI concentration of 100 μg/mL of protein, which is consistent with our knowledge of PI expression in transgenic plants and protein content in plant phloem sap.

2.4.2. Effects of protease inhibitors on A. ervi development

To study the effect of PIs on A. ervi development, neonate aphids were fed for four days on OCI-100, SbBBI-100 and control diets. Host infestation was performed by placing a single mated A. ervi female in a cylinder of artificial diet with 25 five-days-old nymphs for 4 h, then aphids were kept on their respective diet until mummification. Mummies were collected on the day of formation and isolated into gelatin capsules until adult emergence. Fifteen replicates were set up for each treatment.

We recorded the percentage of mummification (i.e. the ratio between the number of mummies formed and the number of aphid nymphs submitted to parasitisation) and of emergence (i.e. the ratio between the number of emerged parasitoids and the number of mummies formed) were calculated. Larval and nymphal development time were defined as the period from host infestation to mummification, and from mummification to adult emergence, respectively. Newly emerged adult parasitoids were sexed. They were then isolated in Petri dishes (5.5 cm diameter) provided with a filter paper imbibed with water until death to evaluate their resistance to starvation. The tibia length was measured under a binocular microscope to estimate the adult size. Experiments were carried out under the same conditions as described above.

2.4.3. Effects of protease inhibitors on A. ervi adult survival

Seventeen one-day-old females were placed in ventilated plastic boxes (17 × 11 × 7 cm) and allowed to feed for 4 h daily on a cotton wool imbibed with sugar-control or BSA100 or OCI100 or SbBBI100. Dead insects were counted and removed every day. Three replicates were carried out under controlled conditions (T = 20 ± 0.5 °C; RH = 60 ± 5%; 16L/8D).

2.5. Determination of A. ervi digestive protease activity

2.5.1. Enzyme preparation

Four day-old aphids issued from synchronised females were placed on cut potato leaves and parasitised by A. ervi females. Third instar parasitoid larvae were extracted by dissecting aphids in 0.15 M NaCl under a binocular microscope at 4 °C. Ten whole parasitoid larvae or 10 dissected larval or adult guts were placed in 30 μL 0.15 M NaCl buffer. Each sample was immediately homogenised on ice then centrifuged at 5000g at 4 °C for 15 min. Supernatants were aliquoted and stored at −20 °C. Adult guts were dissected out of cold-anaesthetised females and extracts made according to
the above procedure. Total protein content of parasitoid extracts was determined by the Bradford (1976) method using BSA (1 µg/mL) as standard protein.

2.5.2. Electrophoresis assay

Whole larvae, larval or adult gut extracts were diluted 2-fold in Laemmlli buffer containing 2% (v/v) β-mercaptoethanol. The samples were separated in a 10% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) copolymerised with 0.05% gelatin. After separation at 100 V at 4 °C, the gel was washed in 2.5% Triton X-100 for 1 h and rinsed twice with distilled water. Subsequently the gel was incubated overnight at 37 °C in 50 mM Tris–HCl buffer pH 8 containing 150 mM NaCl, 5 mM CaCl2 and 2 mM DTT then stained with Coomassie R-250 brilliant blue solution. Indeed, preliminary experiments (data not shown) revealed that the optimal pH range for the protease activity was 8–9 for the larvae and 7–8 for the adults.

2.5.3. Inhibition assays

Electrophoresis assay showed that whole larva and larval gut extracts presented the same proteolytic profile (Fig. 1). Thus, protease activity of both A. ervi adult guts and whole larvae was quantified using the fluorescent protein casein as substrate, at a final concentration of 0.15 mg/L. Inhibition assays were carried out in 0.1 M Tris–HCl pH 8. An equivalent of 1.5 larvae or 5 adult guts per well was incubated for 15 min at ambient temperature with inhibitors, before adding the casein substrate. Fluorescence was measured every 2 min during 60 min at 37 °C on a LabSystems Fluoroskan II fluorimeter at 538 nm after an excitation at 485 nm. The class of chemical and protein inhibitors, and final assay concentrations tested are presented in Table 4.

All assays were performed in triplicate for larvae and duplicate for adults, with appropriate controls. The inhibition of the proteolytic activity was expressed relatively to the control. Inhibitors were dissolved in milli-Q water (E-64, pefablock, EDTA, SbBBI and OCI) or DMSO (pepstatin A and chymostatin).

2.6. Transfer of protease inhibitors from artificial diet to parasitoids and aphid honeydew

2.6.1. Aphids and parasitoids on artificial diet

Aphids were reared on the following diets OCI-100, OCI-250, SbBBI-100, SbBBI-500 and control. On the fifth day, aphids were parasitised as described before. At the beginning of the aphid cuticle melanisation (corresponding to the 3rd instar of the parasitoid larva), a group of aphids were dissected to collect parasitoid larvae. The remaining aphids were kept on their respective diet until mummification. Mummies were collected on the day of formation and placed in a Petri dish until adult parasitoid emergence.

2.6.2. Honeydew collection

Nitrocellulose membrane was placed during 24 h under plastic cylinder containing 20 aphids fed during three days with OCI-100, SbBBI-100 or control. The same number of aphids was kept in each plastic cylinder (two repetitions per diet). Nitrocellulose membranes were then placed between two blotting papers and kept at 4 °C until use.

2.6.3. Immunoassay blot

Detection of OCI and SbBBI in protein extracts from parasitoids was performed by western analysis using anti-OCI and anti-SbBBI antibodies, respectively. Protein samples were extracted by homogenising parasitoids (larvae and adults) in 50 mM Tris–HCl, (pH 6.8) containing 1% SDS (3 μL of buffer per individual). Laemmli buffer containing 2% β-mercaptoethanol was then added and the samples were heated at 95 °C during 5 min. Samples were centrifuged for 15 min at 5000g at 4 °C. The supernatants were electrophoresed in 10% SDS–PAGE then blotted onto nitrocellulose membrane according to Towbin et al. (1979). OCI and SbBBI diluted in sample buffer were used as controls. After electrotransfer at 100 mA, the membrane was blocked for 1 h at room temperature with a solution of 5% (w/v) skimmed milk in TBS (10 mM Tris–HCl pH 7.5, 0.1 M NaCl) containing 0.25% (v/v) Tween-20. OCI or SbBBI antibodies (1:500) and the peroxidase-coupled goat anti-rabbit IgG (1:50,000) were used to hybridise the membrane. Immunostained bands were detected by

![Fig. 1. Zymogram of the gelatinolytic activity of whole larvae, larval and adult gut extracts from Aphis ervi (6 µg of protein of each sample was submitted to electrophoresis).](image)
chemiluminescence (ECL) according to the manufacturer’s instructions. The nitrocellulose membrane containing honeydew droplets excreted by aphids were treated as described before except that the secondary antibody concentration used was 1:1000. In our conditions the detection limit of the western blots was 10 ng for SbBBI and 0.1 ng for OCI.

2.7. Statistical analysis

Statistical analyses were done using STATISTICA 5.5 software (StatSoft®, Tulsa, Oklahoma, USA). The effects of PIs on *M. euphorbiae* size and parasitism success of *A. ervi* were analysed using Kruskal-Wallis-ANOVA. When a significant effect was found the Mann-Whitney-U test was performed to compare the means. The significance level (x) of the Mann-Whitney-U test was corrected according to the number of comparisons, using the Bonferroni adjustment: $x' = x/k$ with $x = 5\%$ nd $k =$ number of comparisons. The effects of PIs on *A. ervi* development were analysed using Mann-Whitney-U test. The results are reported as mean ± standard error (SE).

3. Results

3.1. Effects of protease inhibitors on *M. euphorbiae* size

*M. euphorbiae* fed for five days on diet containing OCI were significantly smaller in both length and width compared to those feeding on control or SbBBI diets (Table 1) (length: OCI versus control $U = 113.0$, $n = 30, P = 0.000$; OCI versus SbBBI $U = 233.5$, $n = 30, P = 0.001$; width: OCI versus control $U = 104.0, n = 30, 30, P = 0.000$; OCI versus SbBBI $U = 214.5, n = 30, 30, P = 0.000$). However, no significant effect on aphid size was observed for those fed on SbBBI diet compared to control (length: SbBBI versus control $U = 113.0, n = 30, 30, P = 0.000$; width: SbBBI versus control $U = 104.0, n = 30, 30, P = 0.000$).

![Table 1](image)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OCI-100</th>
<th>SbBBI-100</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (μm)</td>
<td>1704.4±23.6 (a)</td>
<td>1406.7±40.3 (b)</td>
<td>1600.7±38.1 (a)</td>
<td>$H = 25.7, df = 2, P = 0.000^*$</td>
</tr>
<tr>
<td>Width (μm)</td>
<td>694.1±10.8 (a)</td>
<td>565.2±15.7 (b)</td>
<td>650.4±15.1 (a)</td>
<td>$H = 28.1, df = 2, P = 0.000^*$</td>
</tr>
</tbody>
</table>

Different letters indicate that treatments were significantly different at $x' = 0.017$ (Mann-Whitney-U-test). The acceptance level of statistical significance was $P < 0.05$ (Kruskal-Wallis test). *: Indicates significant differences at $P < 0.05$. ($n$) correspond to the number of replicates. SE: standard error.

3.2. Effects of protease inhibitors on *A. ervi* development

Percentage of mummification was significantly reduced in the case of parasitised OCI-100 intoxicated aphids (Table 2). No adult parasitoid emerged from these aphids. In contrast, percentages of mummification and emergence were not affected when parasitoids developed on SbBBI-intoxicated aphids. The sex ratio of parasitoid offspring from SbBBI-intoxicated aphids was biased towards males whereas mostly females emerged from control aphids. The larval and nymphal development time, and resistance to starvation of offspring developed in SbBBI-100 intoxicated aphids were not affected by the presence of this PI. Only the female size was significantly reduced (Table 3).

![Table 2](image)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OCI-100</th>
<th>SbBBI-100</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mummification (%)</td>
<td>29.3±4.7 (a)</td>
<td>15</td>
<td>28±4.1 (a)</td>
<td>$H = 25.3, df = 2, P = 0.000^*$</td>
</tr>
<tr>
<td>Emergence (%)</td>
<td>40.4±4.5 (a)</td>
<td>14</td>
<td>48.4±5.7 (a)</td>
<td>$H = 13.5, df = 2, P = 0.001^*$</td>
</tr>
<tr>
<td>Sex ratio (% females)</td>
<td>83.7±6.3</td>
<td>13</td>
<td>33.8±10.4</td>
<td>$U = 24.5, df = 1, P = 0.002^*$</td>
</tr>
</tbody>
</table>

Different letters indicate that treatments were significantly different at $x' = 0.017$ (Mann-Whitney-U-test) (mummification %: SbBBI versus control $U = 103.5, n = 15, 15, P = 0.709$; OCI versus control $U = 14.5, n = 15, 15, P = 0.000$; SbBBI versus OCI $U = 3.5, n = 15, 15, P = 0.000$; emergence %: SbBBI versus control $U = 81.5, n = 15, 14, P = 0.305$; OCI versus control $U = 3.0, n = 6, 14, P = 0.001$; SbBBI versus OCI $U = 3.0, n = 15, 6, P = 0.001$). The acceptance level of statistical significance was $P < 0.05$ (Kruskal-Wallis test). *: Indicates significant differences at $P < 0.05$. ($n$) correspond to the number of replicates. SE: standard error.
Table 3
Different parameters (means ± SE) of *Aphidius ervi* development on *Macrosiphum euphorbiae* fed with or without protease inhibitors

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>SbBBI-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male adult size (μm)</td>
<td>7.2 ± 0.1</td>
<td>7.2 ± 0.1</td>
</tr>
<tr>
<td>Female adult size (μm)</td>
<td>7.2 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>Nymphal development time (days)</td>
<td>12.6 ± 0.4</td>
<td>12.6 ± 0.2</td>
</tr>
<tr>
<td>Male adult size (μm)</td>
<td>55.2 ± 1.3</td>
<td>55.7 ± 1</td>
</tr>
<tr>
<td>Female adult size (μm)</td>
<td>63.8 ± 1.1</td>
<td>58.9 ± 1.3</td>
</tr>
</tbody>
</table>

The acceptance level of statistical significance was *P* < 0.05 (Mann-Whitney-U-test). #: Indicates significant differences at *P* < 0.05. ns: indicates no significant differences at *P* < 0.05. (a) correspond to the number of parasitoid offspring. SE: standard error.

Table 4
Effects of chemical inhibitors on the proteolytic activity of *Aphidius ervi*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target proteases</th>
<th>Final assay concentration</th>
<th>Inhibition (% of control)</th>
</tr>
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<tbody>
<tr>
<td>Chemical inhibitors</td>
<td></td>
<td></td>
<td>Larvae</td>
</tr>
<tr>
<td>E-64</td>
<td>Cysteine</td>
<td>500 μM</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Pefablock</td>
<td>Trypsin and Chymotrypsin</td>
<td>10 mM</td>
<td>89 ± 0</td>
</tr>
<tr>
<td>EDTA</td>
<td>Metallo-proteases</td>
<td>10 mM</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>Aspartate</td>
<td>100 μM</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>Chymostatin</td>
<td>Chymotrypsin and Cysteine</td>
<td>300 μM</td>
<td>70 ± 0</td>
</tr>
</tbody>
</table>

Protein inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target proteases</th>
<th>Final assay concentration</th>
<th>Inhibition (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SbBBI</td>
<td>Trypsin and Chymotrypsin</td>
<td>100 μg/mL</td>
<td>86 ± 1</td>
</tr>
<tr>
<td>OCI</td>
<td>Cysteine</td>
<td>100 μg/mL</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

Assays were performed in triplicate for whole larvae and in duplicate for adult gut at pH 8. Results are expressed as means ± SE (standard error). An equivalent of 1.5 larvae (26 μg of protein) or an equivalent of 5 adult guts (3 μg of protein) were incubated for 15 min at ambient temperature with inhibitors, before addition of substance to start the reaction.

3.4. Determination of *A. ervi* digestive protease activity

The analysis of the zymogram (Fig. 1) showed that whole larvae and larval gut extracts presented the same proteolytic profile. Four major bands of gelatinolytic activity were resolved at approximately 21, 29, 50, and 108 kDa then whole larvae were used in the following experiments instead of larval guts. In contrast, proteolytic profile of adult guts revealed only two bands at 21 and 70 kDa.

Chemical inhibitors were first used to determine classes of *A. ervi* digestive proteases (Table 4). Both serine PIs, pefablock and chymostatin, induced a significant inhibition of larval protease activity (89% and 70% of inhibition, respectively). However, E-64, a highly specific cysteine PI, caused only a slight inhibition (10%). Intermediate proteolytic activity inhibition was measured with EDTA and pepstatin A (16% and 28%, respectively).

The four inhibitor protease classes were also used to inhibit the proteolytic activities of adult gut extracts. The serine PIs, pefablock and chymostatin induced the higher levels of inhibition, of 55% and 53% of the proteolytic activity, respectively. The cysteine inhibitor, E-64, induced 37% inhibition of proteolytic activity. The aspartyl PI, pepstatin A, and the metallo-PI, EDTA, induced weaker inhibition of 18% and 6%, respectively.

The protein inhibitors of serine and cysteine proteases, SbBBI and OCI, respectively, were used against the protease activities of *A. ervi* whole larvae and adult glucose-fructose diet (14.7 ± 0.6 days; *n* = 51) (*H* = 2.8, df = 3, *P* = 0.419).
The differences of inhibition levels matched those observed with the corresponding chemical inhibitors. Indeed, the assays performed with these PIs showed that SbBBI induced a much higher inhibitory effect compared to OCI. The trypsin–chymotrypsin inhibitor SbBBI (100 μg/mL) inhibited 86% and 69% of larvae and adult protease activities, respectively. The cysteine PI OCI (100 μg/mL), presented only 10% and 30% inhibition against whole larvae and adult gut digestive protease extracts, respectively.

3.5. Detection of protease inhibitors by immunoassays analysis

Western blot analysis showed that the primary antibodies recognised SbBBI (0.1 μg) and recombinant Escherichia coli OCI (0.01 μg) (Fig. 2A and B lane 1). As expected, in extracts from parasitoids developed in aphids fed with control diet no hybridised band could be observed (lanes 2 and 3). Extracts of parasitoid larvae dissected out from aphids fed with SbBBI-100 and SbBBI-500 showed the presence of this PI (8 kDa, Fig. 2A lanes 4 and 6). Whereas, no hybridisation occurred in the adult extracts (lanes 5 and 7). When host aphids were fed with OCI diets, western blots did not reveal any staining in extracts of parasitoid larvae (Fig. 2B). No adult emerged from OCI-intoxicated aphids.

Dot blot analysis showed the presence of SbBBI and OCI in the honeydew produced by aphids fed with SbBBI-100 and OCI-100 diets (Fig. 3).

4. Discussion

Our study demonstrated that SbBBI had a sublethal effect on A. ervi development and offspring (sex ratio, female size), whereas oryzacystatin I OCI prevented parasitoid development (mortality). However, the direct exposure to either PI during adult food intake did not affect female’s survival.

The comparison of the larval and adult protease profiles showed that four different gelatin hydrolysis bands were observed in the extracts of larval gut, and only two gelatinolysis band in adult gut. The parasitoid larvae have a larger proteolytic activity spectrum compared to adults. This difference corresponds to contrasted lifestyles of both stages of A. ervi. The larva develops entirely into the aphid body, consuming host fluids and tissues exclusively (de Eguileor et al., 2001), whereas the adult feeds only on carbohydrate-rich sources like floral and extrafloral nectar and honeydew (Hågvar and Hofsvang, 1991). As the result of different physiological needs and ecology between larvae and adults, the risk and the consequence of exposure to PIs are different.

SbBBI was detected in parasitoid larvae developed on M. euphorbiae reared on artificial diet containing this entomotoxin, and in honeydew. Therefore, A. ervi was exposed to this serine PI during both larval development and adult stage. Nevertheless, this PI was not revealed in adult parasitoids. SbBBI ingested by parasitoid larvae could have been metabolised due to high proteolytic activity known in larval instars of endoparasitoids. Alternatively, SbBBI could have been excreted in the form of meconial pellets just prior to pupation. This fact was reported for A. ervi developed on M. persicae reared on lectin-enriched artificial diet (Couty et al., 2001b).

The study of SbBBI direct effect on A. ervi showed that the protease activities revealed in the whole larvae...
or adult gut extracts were highly inhibited by this PI. A. ervi proteases were also inhibited by the chymostatin, which is a common inhibitor of both chymotrypsin and cysteine proteases. Nevertheless, E-64 did not inhibit the digestive proteolytic activity, suggesting that the major A. ervi proteases belong to the serine protease class and are chymotrypsin-like. Such serine protease-based digestive metabolism was previously shown in the aphid parasitoid A. abdominalis (Azzouz et al., 2005), in the larval stage of the eulophid ectoparasitoid Eulophus pennisicornis (Down et al., 1999) and in the honeybee (Burgess et al., 1996). Our results about digestive proteases of the parasitoid and their sensitivity to SbBBI indicate a potential risk of toxicity of SbBBI at the 100 μg/mL concentration (0.1% of total soluble proteins).

When A. ervi developed on SbBBI-intoxicated aphids, percentages of mummification and emergence were not affected. The former depends on percentage of parasitised aphids (host-acceptance) and on successful larval development. Thus, parasitoid females accepted SbBBI-intoxicated aphids as well as control ones, at least in our non-choice experimental procedure. The larval and nymphal developmental times of both males and females were not affected by the presence of SbBBI in the aphid. The resistance to starvation of parasitoids emerged from aphids reared on SbBBI supplemented diets was unaffected, although the emerging females were significantly smaller. In addition, the proportion of female progeny developed on SbBBI-intoxicated aphids was significantly lower compared to the control. Lower female size and male-biased sex-ratio could be explained by the reduction of aphid size (Cloutier et al., 1981; King, 1987). In fact, a 6% reduction in aphid size was observed in a complementary experiment. Host-size mediated effect on parasitoid size and sex-ratio was also reported by Couty et al. (2001a), when A. abdominalis developed in GNA (Galanthus nivalis agglutinin) intoxicated aphids. In addition, our results on A. ervi proteases indicate that SbBBI may directly affect the parasitic larvae by binding to digestive proteases, thus impairing insect protein digestion. It could also affect haemolymph composition, and thus its nutritional quality for the endoparasitic larvae.

The direct exposure to SbBBI100 during adult food intake did not affect female’s longevity, while this PI induced 69% inhibition of adult digestive protease activity. Even highest concentration of SbBBI (500 μg/mL) did not reduce A. ervi female’s survival (data not shown). Fecundity was not directly assessed in the present study, but the reduction of female size could have important effects on the fecundity (Visser, 1994; Lauzière et al., 2000). In addition, the lower proportion of female in the progeny could affect the parasitoid population (Cloutier et al., 2000a). In another case, a 5% size reduction of the aphid Metopolophium dirhodum induced by partially resistant wheat cultivars led to decreased weight of the braconid parasitoid Aphidius rhopalosiphi by 34% and fecundity by 26% (van Emden, 1995). So, sublethal effects of SbBBI-intoxicated aphids on A. ervi induced an alteration of parasitoid fitness.

Regarding OCI, it could not be detected in parasitoid larvae whatever the OCI concentration used to feed host aphids. The same result was found with A. abdominalis developed in OCI-intoxicated aphids (Azzouz et al., 2005). Previous study revealed that OCI bound to aphid proteins and the free form was not detected anymore in this insect (Azzouz et al., 2005). This explains the absence of OCI detection in endoparasitoid larvae. We found OCI in honeydew excreted by M. euphorbiae reared on artificial diet supplemented by this PI. This is in accordance with the detection of OCI in honeydew of M. persicae reared on OCI-expressing transgenic oilseed rape (Rahbé et al., 2003a). The study of OCI direct effect on A. ervi showed that the digestive proteolytic activities revealed in the whole larvae or adult gut extracts were slightly inhibited by this PI (10% and 30% for larvae and adult, respectively). These results indicate that the direct risk of exposure of A. ervi to the cysteine PI OCI was a priori low. Adult A. ervi survival was not affected after exposure to PIs during food intake. Nevertheless, our bioassays showed that A. ervi could not develop to the adult stage in OCI-intoxicated aphids (few mummified aphids and no adult emergence). Such drastic effects on A. ervi can be explained by reduced host survival and quality. Indeed, a complementary experiment showed that the size of M. euphorbiae reared on OCI supplemented diet (OCI-100) was significantly reduced by 17% and the nymphal survival was significantly altered (80% of mortality) (Azzouz et al., 2005). The adverse effects were not the consequence of a direct action of OCI on A. ervi by binding to digestive proteases, since the parasitoid has a low cysteine digestive proteases activity. However, detrimental effects of OCI could come partly from the disruption of non-digestive parasitoid proteases (Faktor and Raviv, 1997). Indeed, OCI could reach non-digestive proteases of the immature parasitoid because immuno-histological studies performed on M. persicae showed that OCI crosses the midgut epithelium and reaches the hemolymph, where it accumulates in oenocytes and bacteriocytes. As protease activity is intense during metamorphosis of the parasitoid larvae, it might be impaired even by very small amount of the PIs, leading to nymphal mortality.

We investigated effects in the case of controlled exposure to PIs in laboratory systems. What happens with PI-expressing transgenic plants? Neither the fitness of A. ervi females, nor the sex ratio of their offspring were affected when the parasitoid developed on aphids feeding on OCI-potatoes (Cowgill et al., 2004). The lack of effects on parasitoid could be due to a very low level
of the OCI variant (OCIΔD86) in the phloem sap. In fact, immunoassay experiments showed that OCI was not detected in aphids that fed on OCI-potatoes (Cowgill et al., 2004). Another study showed that Aphidius nigripes exhibited an increased size and fecundity when developed on M. euphorbiae feeding on OCI-expressing potatoes (Kennebec-OCI) (Ashouri et al., 2001). But this probiotic effect may not be related to OCI because the authors did not check OCI level in their plants, and transgene expression is difficult to control in transgenic potatoes due to tetraploidy. The risk assessment study by Schuler et al. (2001) using M. euphorbiae reared on OCI-expressing oilseed rape (Drakkar) did not reveal any adverse effect on another Aphidiidae parasitoid, Diaprius cerasi. Nevertheless, the OCI expression level in the phloem of the tested plants was twice higher (0.2% of total protein) than the dose used in our experiment (Rahbé et al., 2003a). These data highlight the difficulty to control PI expression in transgenic plants, which can vary according to transgenic lines, plant physiology and tissue nature (De Leo et al., 1998, 2001; Cloutier et al., 2000b; Marchetti et al., 2000; Cowgill et al., 2004). With regard to a risk of PIs effect when adult parasitoids feed on honeydew, it can be considered as negligible, because we found that PIs ingestion by adult A. ervi was not harmful. However, honeydew that contains transgene products such GNA (Galanthus nivalis agglutinin) could pose a risk to honeydew feeding parasitoids (Romeis et al., 2003; Bell et al., 2004). PIs could not be detected in the nectar of transgenic plants containing genes encoding these proteins, especially when the gene was under the control of the CaMV 35S promoter (Malone and Burgess, 2000; Couty et al., 2002). Thus, adult parasitoids could not be intoxicated by nectar of PIs transgenic plants.

Considering unreliability of PIs expression in currently available transgenic potatoes, we worked with controlled amounts of PIs delivered via artificial diets, in the objective to get reliable effects on the parasitoid. It allowed us to estimate the consequence of an exposure to plant PIs and the sensitivity of the aphid parasitoid A. ervi to these entomotoxins, by combining immunological, biochemical and biological approaches. First it pointed out that only immature stages are affected by PIs. Secondly, it documented two different modes of effect, according to the nature of the PIs and both host and parasitoid susceptibility. A. ervi development disruption appears the consequence of its host susceptibility to OCI, whereas SbBBI only induced sublethal effects on the parasitoid, possibly due to both direct action on the parasitoid susceptible proteases, and host-mediated action through size reduction.

Now, in order to better predict side effects of PIs transgenic plants on the third trophic level, analysing effects of a range of PIs doses is one way to face variable expression in planta. Our study shows that such analyse can be restricted to the preimaginal development and offspring fitness parameters. To predict effects on a longer term, it will be necessary to characterise effects on offspring fitness, by studying the reproductive potential at the second generation of parasitoids. This data, together with the improvement of PIs transgenic plants, are needed to sort out a key issue for sustainable agriculture, i.e. the compatibility between transgenic insect-resistant plants and other components of integrated pest management (IPM), to get durable and effective pest resistance.

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