The proregion of papaya proteinase IV inhibits Colorado potato beetle digestive cysteine proteinases

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Abstract Three distinct digestive protease systems were induced in larvae of the herbivorous pest, Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say), and used as a model to assess the ability of the proregion of papaya proteinase IV (PPIV; glycyl endopeptidase, EC 3.4.22.25) to act as an inhibitor of insect digestive cysteine proteinases. As shown by gelatin/PAGE and complementary inhibition assays, a recombinant form of the proregion produced in *Escherichia coli* inhibited a fraction of the insect proteases also inhibited by the well-characterized inhibitor of cysteine proteinases, oryzacystatin I (OCI). In contrast with OCI, the inhibitory potency of the proregion was affected by an increase of the temperature, suggesting a certain alteration of its structural integrity by the insect non-target proteases. This apparent susceptibility to proteolysis was confirmed by SDS-PAGE, after challenging the proregion with the different insect extracts. As seen on gel, selective inhibition of the insect aspartate proteinase, cathepsin D, with the inhibitor pepstatin A preserved the activity of the proregion against cysteine proteinases by preventing its hydrolysis. Taken together, these observations suggest the potential of plant protease proregions as regulators of cysteine proteinases in biotechnological systems, and show the ability of protease inhibitors to preserve the integrity of 'companion' defense-related proteins from the action of insensitive proteases in target pests.

Key words: Insect digestive protease; Cysteine proteinase; Papaya proteinase IV proregion; Glycyl endopeptidase proregion; Proteinase inhibitor; Colorado potato beetle; *Leptinotarsa decemlineata* Say

1. Introduction

The importance of extracellular proteases in plant/pest and plant/pathogen interactions is well-recognized [1], and control strategies based on their inhibition with selective inhibitors have been proposed as a way to control herbivorous insects, parasitic nematodes, and microbial pathogens [2–4]. Although the exact metabolic functions altered by plant protease inhibitors remain to be elucidated in most cases, the importance of proteolytic enzymes in target pests and pathogens appears obvious. The repressive effects of protease inhibitors on insect growth or fecundity, for instance, have been described for several species [5], and the implication of microbial extracellular proteases in plant pathogenic processes has been suggested in several instances [6,7]. Based on these data, the use of protease inhibitor-expressing transgenic plants has been proposed as a potential mean of protecting crops from their natural enemies, and several plants of economic importance have been genetically engineered with inhibitor-encoding cDNA sequences during the last ten years [5,8,9].

Despite these important advances, the general usefulness of recombinant protease inhibitors in plant protection still remains equivocal. The inhibitory range of protease inhibitors is usually limited to proteinases in one of several mechanistic classes, leaving free proteases in the surrounding medium after inhibition. Possibly due to coevolution processes, the inhibitory spectrum of plant protease inhibitors against herbivorous pest proteinases is even more limited, being restricted in several cases to the subclass level [10–12]. The occurrence of insensitive proteases in target pests, that may allow physiological compensation of inhibited proteolytic functions [13], may also challenge the structural integrity of certain inhibitors [14]. The ability of cysteine-type protease inhibitors to retain their structural integrity in the presence of insect insensitive proteases, for instance, was assessed with human stefin A and two inhibitors from rice, oryzacystatin I (OCI) and oryzacystatin II (OCII) as model inhibitors [15,16]. While OCI remained stable in the presence of insect insensitive proteases, OCII and the human inhibitor were subjected to extensive hydrolysis, gradually leading to a complete loss of their inhibitory activity.

Understanding the dynamic interactions implicating plant protease inhibitors and the digestive proteases of herbivorous pests clearly appears important to correctly assess the actual usefulness of extracellular protease inhibition in plant protection. From a practical point of view, the development and the identification of alternative or complementary inhibitors is also important to achieve broad-spectrum inhibition of pest protease systems and thus minimize the occurrence of compensatory or degradation processes in target pests [14,17]. Several strategies are currently considered for the improvement of protease inhibitor-based control approaches, including: (i) the improvement of inhibitor binding characteristics by site-directed mutagenesis [3], (ii) the isolation of effective inhibitor variants by phage display [17,18], (iii) the isolation of novel, stress-induced inhibitors from plant tissues [19], (iv) the use of insect protease inhibitors exhibiting high affinity for insect digestive proteinases [20], and (v) the use of insect regulatory propeptides specific to their cognate proteinase [21]. In this study we assessed the ability of a plant protease proregion, the proregion of papaya proteinase IV (PPIV; glycyl endopeptidase, EC 3.4.22.25) [22], to act as an inhibitor of herbivorous pest digestive cysteine proteases, and to remain stable in the presence of non-target, insensitive proteases. Diet-related variants of the well-characterized digestive protease system of the insect pest, Colorado potato beetle (CPB;

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Abbreviations: CPB, Colorado potato beetle; OCI, oryzacystatin I; OCII, oryzacystatin II; PPIV, papaya proteinase IV

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Leptinotarsa decemlineata Say) were used as an insect model for the inhibition assays.

2. Materials and methods

2.1. Reagents

Azocasein, bovine serum albumin (BSA), L-cysteine, gelatin (porcine type A), pepstatin A, trichloroacetic acid and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Electrophoretic reagents were from Bio-Rad (Richmond, CA, USA). All other reagents were of the highest purity commercially available.

2.2. Inhibitors

Pepstatin was dissolved in methanol to a 1-mM final concentration. The proregion of PPIV was expressed as a soluble polypeptide in *Escherichia coli* and purified by chromatography on a 1-ml Hitrap Q column (Pharmacia, Uppsala, Sweden), as described previously [22]. Recombinant OCI was produced in *E. coli* JM109 using the glutathione *S*-transferase gene fusion system, with the plasmid pGEX3X-OCI as the expression vector [23]. Purity of the recombinant protein inhibitors was controlled by standard sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [24]. Inhibitor concentration in the resulting preparations was adjusted to 1.5 mg/ml with 50 mM Tris, pH 8.0. Protein concentrations were determined according to Bradford [25], with BSA as a protein standard.

2.3. Insect digestive proteases

Third-instar CPB larvae reared on greenhouse-grown potato plants (cv. Kennebec) were placed at 22°C in 100-ml aerated plastic arenas containing water-saturated cotton sticks and a moist filter paper, under a 16-h/8-h (L:D) photoperiod provided by cool white fluorescent lights. The fifth leaves of either 'control' potato (cv. Kennebec) or OCI-expressing transgenic potato (cv. Kennebec) [9] were supplied to the insects at 24-h intervals over a three-day period. In parallel, third instars were starved for three days under the same growth conditions. Each experiment was repeated three times, with 10 larvae in each arena. The insect digestive soluble proteases were then extracted as described previously [26], in a 100-mM citrate phosphate buffer, pH 6.0, containing 10% (w/v) ethylene glycol. After centrifuging the mixture at 4°C for 30 min at $17\,000\times g$, the supernatant was passed through a Sephadex G-25 column to remove low molecular-weight compounds, and used as a source of proteases for subsequent analyses. Protein content in the extracts was determined according to Bradford [25], with BSA as a standard.

2.4. Gel protease assay

The insect digestive proteinases were visualized by mildly-denaturing gelatin/SDS-PAGE [27]. The protein extracts were fractionated into 0.1% (w/v) SDS-10% (w/v) polyacrylamide slab gels containing 0.1% (w/v) gelatin. Samples containing 0.25 unit of protease activity (see below) were first subjected to electrophoresis at 4°C using a Bio-Rad Mini-Protean II unit. After migration at 200 V, the gel was transferred to a 2.5% (v/v) aqueous solution of Triton X-100 for 30 min at room temperature to allow enzyme renaturation, and then placed in an assay (proteolysis) buffer (100 mM citrate phosphate, pH 6.0, 0.1% (v/v) Triton X-100, 5 mM L-cysteine) for 3 h at 37°C. Proteolysis was stopped by transferring the gel into a staining solution (0.1% (w/v) Coomassie Brilliant Blue in 25% (v/v) isopropanol/10% (v/v) acetic acid), and the proteases (gelatinases) were visualized as clear bands against a dark background.

2.5. Protease and protease inhibitor assays

Total protease activity in the extracts was determined according to a previously described procedure [10], in the presence or absence of inhibitors. An appropriate amount of insect extract (corresponding to 0.25 unit of protease activity) was mixed with 5 μl of inhibitor solution (7.5 μg protein) or 5 μl of 50 mM Tris, pH 8.0 (ctrls), and the total volume of the mixture was adjusted to 50 μl with assay buffer (see above). After a 30-min incubation at 37°C, 50 μl of 2% (w/v) azocasein (1 mg protein) in assay buffer was added to the enzyme/inhibitor solution, and the complete mixture was incubated for 3 h at 37°C. After proteolysis, 300 μl of 10% (w/v) trichloroacetic acid was added to the mixture, and residual azocasein was removed by centrifugation at $13\,000\times g$ for 5 min. The supernatant (350 μl) was added to 300 μl of 1 N NaOH, and the absorbance (A) was measured at 440 nm using a Spectronic 1000 Plus spectrophotometer (Milton Roy, Rochester, NY, USA). The A_{440} of blanks, which consisted of complete mixtures incubated for 0 h, was subtracted from each value. One unit of activity was defined as the amount of insect extract needed to cause an absorbance change of 1.0 in a 1-cm cuvette, under the conditions of the assay. All measures were done in triplicate.

2.6. Stability of the PPIV proregion

Susceptibility of the recombinant proregion to the action of CPB digestive proteases was assessed by incubating the inhibitor with the different insect extracts. Briefly, an amount of insect extract containing 0.125 unit of protease activity was incubated for 60 min at 37°C with 5 μl of the proregion preparation (7.5 μg protein). After incubation, the reaction was stopped by adding an equal volume of SDS-PAGE sample buffer 2X [24] to the enzyme/inhibitor solution, and then placing the complete mixture in a boiling water bath for 3 min. The residual proregion was visualized as a 13-kDa band following 15% (w/v) SDS-PAGE [24]. The protective effect of pepstatin A on the integrity of the propeptide was visualized by adding 1 μl of the pepstatin preparation to the insect proteases/proregion mixture, before the incubation at 37°C.

3. Results and discussion

3.1. The ratio of OCI-sensitive to insensitive cysteine proteinase activity in CPB larvae is drastically increased during starvation

In CPB, most of the digestive protease activity is explained by multiple proteinase forms present throughout development of the insect [28] and presumably belonging to the cathepsin B, cathepsin H, cathepsin D, and chymotryspin protease families [29,30]. This apparent complexity of the insect digestive protease system, which possibly indicates a physiological advantage for the insect of using different proteinase forms with various specificities, also gives it a certain ability to easily adapt its digestive protease metabolism to the presence in the diet of compounds interfering with dietary protein hydrolysis. The nature of the CPB digestive protease system is strongly influenced, notably, by the type of diet ingested [26] and by the presence of protease inhibitors in the host plant tissues [31]. Such compensatory responses of the insect digestive proteolytic metabolism, while providing a good example of the complexity of dietary protein digestion in herbivorous pests, also suggest the usefulness of this system as a tool for studying the complex and dynamic interactions between pest digestive proteases and plant protease inhibitors.

In this study, 'new' proteinase complements were induced in the insect midgut by providing third instars previously reared on control (untransformed) potato plants with OCI/expressing transgenic potato foliage, or by starving the larvae for a three-day period (Fig. 1). In accordance with the occurrence of OCI-sensitive digestive proteinases in CPB larvae [10], the activity of a major proteinase form detected in gel was lower in the extracts prepared from insects supplied with OCI/expressing potato (Fig. 1, arrow 1), demonstrating the effective inhibitory potency of recombinant OCI expressed in planta. In contrast, starved insects overexpressed the OCI-sensitive proteinase, while not expressing most of the insensitive species. A proteinase form absent from the extracts of both potato- and OCI/potato-fed insects (Fig. 1, arrow 2), and from those of eggplant- or tomato-fed insects (not shown) was also detected, suggesting the existence of a starvation-specific digestive proteinase in CPB larvae. Although several questions remain regarding its physiological significance, this starvation-induced protease system, mainly composed of OCI-sensitive

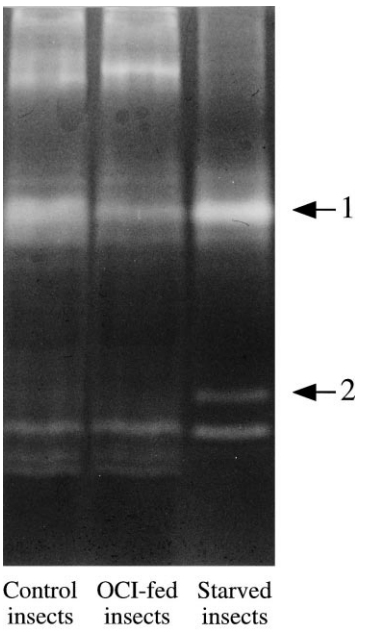

Fig. 1. Detection of CPB digestive proteinase forms after mildly-denaturing gelatin/SDS-PAGE. The arrows show the OCI-sensitive proteinase (arrow 1) and a starvation-specific proteinase (arrow 2).

cysteine proteinase activity, provides an additional argument supporting the usefulness of CPB digestive proteases as a model to study the relative importance of inhibitor-sensitive and insensitive digestive proteases in herbivorous pests, and to assess the potential of novel cysteine proteinase inhibitors in plant protection.

3.2. *The proregion of PPIV inhibits the insect OCI-sensitive digestive proteinase*

In this study, the ability of the PPIV proregion to inhibit insect digestive cysteine proteinases was assessed by measuring its inhibitory activity against the different protease systems described in Fig. 1. In cells, proteinase proregions are thought both to assist folding of the mature enzyme and to prevent uncontrolled proteolysis by acting as inhibitors of their cognate enzyme. After expressing the PPIV propeptide in *E. coli*, Taylor et al. [22] showed that it could inhibit not only PPIV but also other papaya proteinases, including papain. As shown here by standard inhibition assays, the plant proregion also shows affinity for a fraction of the CPB digestive cysteine proteinases (Fig. 2), suggesting that it could actually represent a general inhibitor of papain-like cysteine proteinases. In accordance with the distinct ratios of OCI-sensitive to insensitive proteinase activity in control, OCI/potato-fed and starved insects, the inhibitory spectrum of both OCI and PPIV proregion against the insect proteinases drastically varied depending on the enzyme system assessed. Roughly, the inhibitory spectrum of OCI ranged from ~30–40% of total protease activity for control and OCI-fed insects to more than 80% for the starved insects. Similar inhibitory spectra were noted for the PPIV proregion when the assays were done at 25°C, although they were narrower for assays carried out at 37°C. Interestingly, no complementary inhibition was noted when the proregion and OCI were used in combination, strongly suggesting that the proteinase recognized by the first inhibitor corresponded to the OCI-sensitive cysteine proteinase visualized in gel (Fig. 1, arrow 1), presumably a cathepsin H-like enzyme [10]. Taken together, these observations provide evidence that the inhibitory spectrum of proteinase inhibitors against insect digestive proteinases is determined not only by the basic affinity between these inhibitors and their target enzymes, and by the ability of insects to produce insensitive proteases following ingestion of dietary inhibitors [11,12], but may also be strongly influenced by the physiological status of the target organism submitted to stress conditions such as starvation.

Our results also point out the possible usefulness of plant cysteine proteinase proregions as a tool for the inhibition of cysteine proteinases in biological systems. The narrow activity spectrum of the PPIV propeptide against CPB digestive cysteine proteinases, although showing the limited potential of this inhibitor in CPB control, does not exclude interesting effects on alternative protease systems. Complex dissociation studies involving OCI and OCII as model inhibitors clearly revealed the differential effects of these closely-related plant inhibitors on the extracellular cysteine proteinases of herbivorous pests (e.g. [32]), strongly suggesting the importance to assess the effect of several inhibitors when planning the control of a particular pest. As a 'general' inhibitor of cysteine proteinases, the proregion of PPIV (and possibly those of other cysteine proteinases) could provide an interesting complement to the currently used cysteine-type inhibitors, extending the diversity of inhibitors available for the regulation of cysteine proteinases in various systems. Pest extracellular protease systems composed of a limited number of OCI-sensitive cysteine proteinase species, notably, could represent interesting target enzyme systems for plant cysteine proteinase proregions expressed as recombinant proteins in transgenic plants.

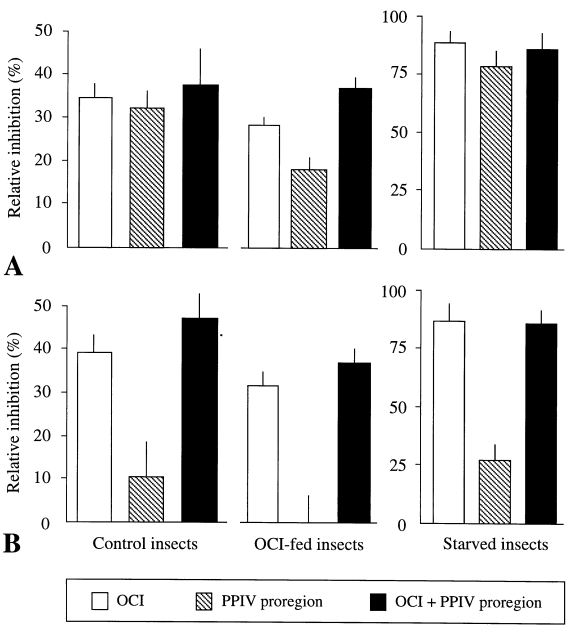

Fig. 2. Inhibition of CPB digestive protease activity by OCI and PPIV proregion, alone or in combination. The assays were carried out at 25°C (A) or 37°C (B) as described in Section 2. Results are expressed as relative inhibitions (%), as compared to controls for which no inhibitor was added. Each bar represents the mean of three values ± S.E.

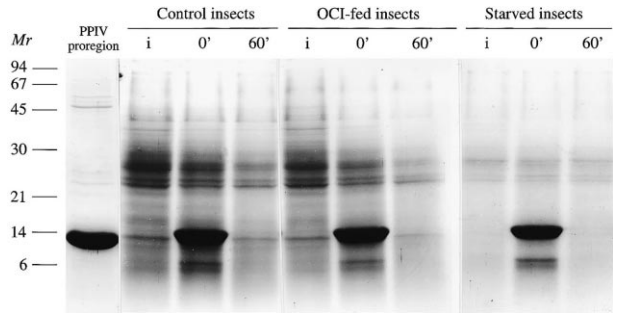

Fig. 3. Response of recombinant PPIV proregion to the action of CPB digestive proteases from potato-fed (control), OCI/potato-fed, or starved insects. After incubation for 60 min at 37°C, the protein mixture was subjected to 15% (w/v) SDS-PAGE and the proregion was visualized as a 13-kDa band following Coomassie Brilliant Blue staining. 7.5 μg of proregion ('time 0' control equivalent) was loaded in each well. i: insect extract; 0′: i+proregion before incubation; 60′: i+proregion after incubation.

3.3. *Pepstatin preserves the inhibitory potency of the PPIV proregion against the insect OCI-sensitive proteinase*

Besides its potential in the regulation of cysteine proteinases, the PPIV proregion could prove useful as a model protein substrate for studying protease-mediated resistance in insects, and for assessing the usefulness of protease inhibitors in the protection of potentially useful, but unstable defense-related proteins [14]. In contrast with OCI, the inhibitory efficiency of the proregion was affected by increasing the temperature from 25°C to 37°C (see Fig. 2), suggesting a certain alteration of its structural integrity by the insect non-target proteases [15,16]. This apparent susceptibility to proteolysis was confirmed by SDS-PAGE, after incubating the proregion at 37°C with the different larval extracts (Fig. 3). Interestingly, the temperature-dependent efficiency and the hydrolysis of the propeptide were noted even with the extracts prepared from starved insects. Considering the negligible importance of OCI-insensitive cysteine proteinases in the corresponding extract (see Figs. 1 and 2), this observation suggested that the extensive hydrolysis of the proregion was due to the presence of a non-cysteine protease in the surrounding medium, presumably the insect cathepsin D-like aspartate proteinase, which is not visualized following gelatin/SDS-PAGE [28] but easily detected by standard assays in mild conditions [28,29].

In agreement with this hypothesis, structural and functional integrity of the PPIV proregion was preserved by the addition of pepstatin A, a specific inhibitor of aspartate proteinases, to the enzyme/inhibitor mixture (Fig. 4). By using a combination of in vitro and diet-based assays, Orr et al. [33] previously suggested that the repressive effect of cysteine proteinase inhibitors against herbivorous insects not only depends on their affinity for target proteinases, but also on their capacity to remain stable in the insect midgut environment. Inclusion of different cystatins in the diet of Southern corn rootworm larvae, for instance, resulted in quite varying effects on growth of the insect, although all inhibitors caused inactivation of the insect digestive proteases in vitro. Interestingly, the simultaneous inclusion of a complementary, non-cysteine protease inhibitor restored both the activity of the less effective cystatins in the insect gut and their antinutritive effect in vivo [33]. While suggesting the importance of digestive cathepsin D-like activity in CPB larvae, the proregion-stabilizing effect of pepstatin provides here additional evidence suggesting the usefulness of protease inhibitors in protecting 'companion' defense-related proteins susceptible to the action of free proteases in the surrounding medium, and in avoiding protease-mediated resistance in herbivorous pests [14].

4. Concluding remarks

This study assessed the potential of a plant cysteine proteinase proregion, the proregion of PPIV, as a tool for the inhibition of cysteine (papain-like) proteinases in biological systems. After developing an insect model system useful in studying the dynamic interactions taking place between plant protease inhibitors and the digestive cysteine proteinases of herbivorous pests, we showed that the PPIV proregion could inhibit not only the cysteine proteinases of papaya, but also those found in the digestive tract of insect pests. Although important questions remain regarding their actual efficiency and stability in vivo, plant protease proregions could thus

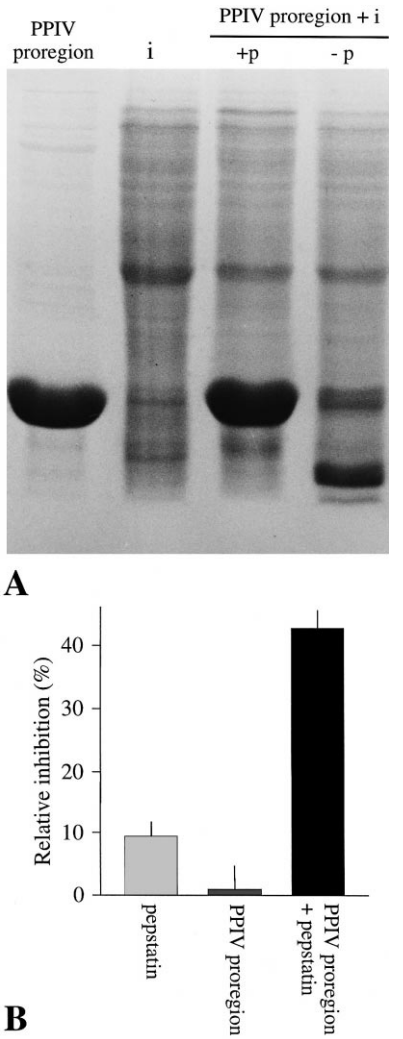

Fig. 4. Stabilization of the PPIV proregion by pepstatin A. A: Susceptibility of the recombinant proregion to the action of potato-fed insects' digestive proteases, in the presence (+p) or absence (−p) of pepstatin A. B: Inhibition of CPB digestive protease activity at 37°C by the PPIV proregion and pepstatin A, alone or in combination. Inhibition assays and stability studies were carried out as described in Figs. 2 and 3. i: insect extract; p: pepstatin A.

prove an interesting complement to the various inhibitors currently used for regulating proteinase activity in complex biological systems. The use of plant proregions in combination with highly specific proregions like those of insect midgut proteinases [21], notably, could prove particularly useful in designing molecular control strategies tailored for the inhibition of specific insect target systems. Works are currently underway to characterize further the stabilizing effect of protease inhibitors on the PPIV propeptide, and to assess the general potential of protease proregions in plant biotechnology.

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