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Selective inhibition of Colorado potato beetle cathepsin H by oryzacystatins I and II

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The use of oryzacystatins I and II, two cysteine proteinase inhibitors naturally produced in rice grains, represents an attractive way for the control of Coleoptera insect pests. The present study was done to analyze the inhibitory effect of recombinant oryzacystatins produced in *Escherichia coli* as fusion proteins against digestive proteinases of the major pest Colorado potato beetle (*Leptinotarsa decemlineata* Say). Both inhibitors had a significant effect on total proteolytic activity, but maximal inhibitions ranged from 20 to 80% for pHs varying from 5.0 to 7.0, respectively. This pH-dependent efficiency of plant cystatins was due to the selective inactivation of potato beetle cathepsin H, as demonstrated by the use of inhibitors with different specificities against cathepsins B and H. These results demonstrate the importance of having an adequate knowledge of insect proteinases specifically recognized by the inhibitors to be used in pest control strategies.

Oryzacystatin I; Oryzacystatin II; Cathepsin H; Colorado potato beetle; *Leptinotarsa decemlineata* Say

1. INTRODUCTION

Transformation of plant genomes with proteinase inhibitor (PI) genes is an attractive approach for the biological control of insect pests [1]. Several studies have demonstrated the repressive effect of serine PIs on insect growth and development [2–4], and tobacco plants transformed with serine PI genes were shown to be resistant to *Heliothis virescens* [5] and *Manduca sexta* [6]. Similarly, growth and development of Coleoptera are delayed by *trans*-epoxysuccinyl-L-(4-guanidino) butane (E-64), a specific cysteine PI (cPI) isolated from *Aspergillus japonicum* [7–9]. As phytophageous Coleoptera generally use cysteine proteinases for protein digestion [10], cPIs of the cystatin superfamily represent potential control proteins for the genetic transformation of several economically important crops. Recent studies showed that oryzacystatin I (OCI), a cPI purified from rice (*Oryza sativa* L.) [11], inhibit proteinases and affect growth patterns of several Coleoptera species [12,13], indicating the potential of this inhibitor for the production of Coleoptera-resistant transgenic plants. Oryzacystatin II (OCII), a second cPI purified from rice and highly specific to cathepsin H [14], also appears to be a good candidate for the production of such resistant plants. However, despite these promising data concerning the potential of cPIs for insect control, little is known about the insect proteinases specifically recognized by these inhibitors.

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After describing the production and purification of active OCI and OCII using the glutathione *S*-transferase (GST) gene fusion system [15], the present study demonstrates the biochemical potential of OCI and OCII to inhibit digestive proteinases of the major pest Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say), which primarily uses cysteine proteinases for protein digestion [7,16]. The specific family of proteinases recognized by the two inhibitors is also identified.

2. MATERIALS AND METHODS

2.1 *Materials*

Azocasein, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), leupeptin, reduced glutathione, glutathione-agarose beads and papain (from papaya latex; E.C.3.4.22.2) were purchased from Sigma (St. Louis, MO). Cathepsin B (from human liver; E.C.3.4.22.1), cathepsin H (from human kidney; E.C.3.4.22.6), cathepsin L (from human liver; E.C.3.4.22.15), factor Xa (from human plasma; E.C.3.4.21.6) and lysozyme (from chicken egg white; E.C.3.2.1.17) were obtained from Calbiochem (La Jolla, CA). The expression vector pGEX-3X was from Pharmacia (Uppsala, Sweden). Electrophoretic reagents were from Bio-Rad (Richmond, CA). All other reagents were of the highest purity commercially available.

2.2 *Construction of expression vectors*

Production and purification of large amounts of active OCI and OCII was achieved using the GST gene fusion system [15]. For OCI, the plasmid pOC26–5′-1 [17] was first digested with *Sma*I and *Xba*I. The 0.6 kb DNA fragment encoding a modified form of OCI [17] was isolated, filled in with dNTP/Klenow, and inserted at the *Sma*I site of pUC19 plasmid. The vector pUC19/OCI was digested with *Bam*HI and *Eco*RI, and cloned into the corresponding sites of the expression vector pGEX-3X. This construct (pGEX-3XOCI) encoded a 43 kDa-fusion protein consisting of GST (26 kDa) and OCI (17 kDa). For OCII, the plasmid pNOC1-1 [14] was digested with *Nco*I and treated

by S1 nuclease. After digestion with *Eco*RI, the 0.5 kb insert encoding OCII was isolated and inserted between the *Sma*I and *Eco*RI cloning sites of pGEX-3X. This construct (pGEX-3XOCII) encoded a 38 kDa-fusion protein consisting of GST (26 kDa) and OCII (12 kDa). The two recombinant plasmids, pGEX-3XOCI and pGEX-3XOCII, were transformed in *E coli* JM 109 for expression of the fusion proteins

2.3. *Expression, purification and cleavage of the fusion proteins*

The expression procedure was adapted from Smith and Johnson [15]. Briefly, 100 ml of an overnight culture of *E coli* was inoculated into 1 liter of LB medium containing 100 μg/ml ampicillin. The culture was incubated for 2 h at 37°C with vigorous agitation, before adding IPTG to 0.5 mM final concentration. After incubation for 16 h at 37°C, cells were harvested by centrifugation at 10,000 × *g* for 10 min, and suspended in 15 ml of 50 mM Tris, pH 8.0, containing 5% (w/v) sucrose, 50 mM EDTA, 5% (v/v) Triton X-100 and 0.025% (w/v) lysozyme. After 5 min on ice, the bacterial lysate was centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant containing soluble proteins was saved.

Purification of the fusion proteins using glutathione-agarose beads was performed according to Guan and Dixon [18]. Cleavage of the fusions with human factor Xa was done according to Nagai and Thogersen [19]. Purity of the protein products was determined by 12% (w/v) SDS-PAGE [20].

2.4. *Midgut proteinase extracts*

Midguts from third instar CPB (5 g) were excised, ground in liquid nitrogen, and extracted in 10 ml of a 100 mM citrate–phosphate buffer, pH 5.5. After incubation on ice for 30 min, the mixture was centrifuged at 13,000 × *g* for 30 min at 4°C. The supernatant was then passed through a Sephadex G-25 column to remove low molecular weight compounds. Protein concentration in the extract was adjusted to 10 mg/ml with extraction buffer.

2.5. *Proteinase assays, proteinase inhibitor assays and determination of K_i constants*

The proteinase assay procedure was adapted from Sarath et al. [21]. Enzyme extracts (5 μl per assay) were incubated with 35 μl of assay buffer (100 mM citrate–phosphate at the appropriate pH, 5 mM L-cysteine, 0.1% (v/v) Triton X-100) and 80 μl of 2% (w/v) azocasein diluted in assay buffer, 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 for 5 min at 13,000 × *g*. The supernatant (350 μl) was then added to 300 μl of 1 N NaOH, and the absorbance (A) was measured at 440 nm using a Spectronic 1,000 Plus spectrophotometer (Milton Roy, Rochester, NY). The A_{440} of blanks, which consisted of complete mixtures incubated for 0 h, was subtracted from each value.

For inhibition assays, proteinase inhibitors were diluted to the appropriate concentrations in the 35 μl of assay buffer of the reaction mixture. The enzyme and inhibitor solutions were allowed to react for 15 min at 37°C before addition of the substrate. K_i constants were determined for papain and cathepsin H using Dixon plots [22].

3. RESULTS

3.1. *Specificities of recombinant inhibitors*

Oryzacystatins I and II were produced and purified using the GST gene fusion system (Fig. 1). Both recombinant inhibitors were shown to be active against cathepsin H and papain, whereas no inhibitions were noted for cathepsins B and L (Table I). These enzyme specificities were similar to those reported for the naturally occurring OCI and OCII [11,14]. The activities of the inhibitors (K_i constants) against human cathepsin H and papain were also comparable to those of natural OCs (Table I).

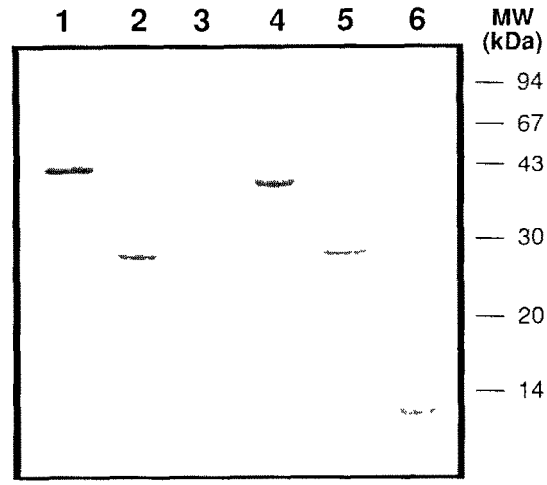

Fig. 1. Purification of oryzacystatins I and II expressed as glutathione *S*-transferase fusion proteins. Lane 1, GST/OCI (fusion protein; MW ~43 kDa); lane 2, GST/OCI, GST (MW ~26 kDa) and OCI (MW ~17 kDa); lane 3, purified OCI; lane 4, GST/OCII (fusion protein; MW ~38 kDa); lane 5, GST/OCII, GST and OCII (MW ~12 kDa); lane 6, purified OCII.

3.2. *Response of CPB digestive proteinases to recombinant OCs*

Fig. 2 shows the effect of recombinant OCI and OCII against CPB digestive proteinases. Increasing concentrations of both inhibitors caused a gradual decrease of proteolytic activity. In either case, the maximal inhibition observed varied with pH (Fig. 2A,B). As CPB larvae mainly use cathepsins B and H for protein digestion [16], the residual activity observed probably corresponds to cathepsin B, which is not affected by OCs (Table I). The pH-dependence of the OC's efficacy is probably due to the relative activity of cathepsins B and H at various pHs. In contrast to CPB cathepsin B, CPB cathepsin H is poorly active at pH 5.0, and its optimal pH is 7.0 [16].

Further, Fig. 2 shows that OCII appears to be a more efficient inhibitor of CPB proteinases than OCI. For instance, the IC_{50} values determined at pH 7.0 were estimated to be ~11 and ~6 μg inhibitor/100 μg midgut protein for OCI and OCII, respectively. This observation is in accordance with the high affinity of OCII for cathepsin H (Table I).

3.3. *OCs specifically inhibit CPB cathepsin H*

The existence of a selective inactivation of CPB cathepsin H by OCI and OCII was assessed by the use of cPIs with different specificities against cathepsins B and H (Fig. 3). The fungal inhibitor E-64, which can inhibit both cathepsin B and cathepsin H [23], caused an almost complete inhibition of CPB digestive pro-

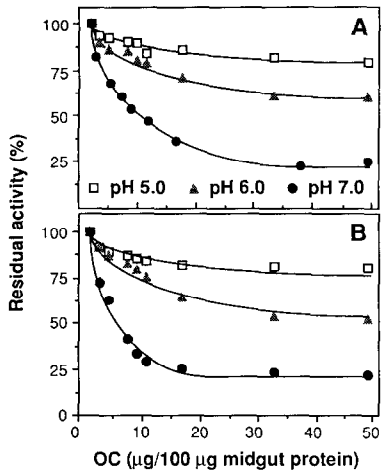

Fig. 2. Response of Colorado potato beetle digestive proteinases to recombinant oryzacystatins I (A) and II (B). Inhibition assays were performed at pHs 5.0, 6.0 and 7.0, on 3rd instar proteinases.

teinases regardless of the pH, whereas OCI, OCII and leupeptin caused partial inhibition. Maximal inhibition by OCI and OCII increased linearly by varying the pH from 5.0 to 7.0. In contrast, a linear decrease was noted for leupeptin, known to be active against cathepsin B but not against cathepsin H [24]. Simultaneous use of OCI (or OCII) and leupeptin led to an almost complete inhibition, as was the case for E-64. Interestingly, the addition of independent inhibitions by OCI (or OCII) and leupeptin gave ~100% inhibition for all the pHs tested, strongly suggesting a selective inactivation of CPB digestive cathepsin H by OCI and OCII. The strong effect of the two plant cystatins and the weak effect of leupeptin at pH 7.0 are apparently due to the relative importance of cathepsin H at this pH [16].

4. DISCUSSION

Recombinant OCI and OCII produced by the GST gene fusion system (Fig. 1) [15] were shown to be as active against cathepsin H and papain as the naturally occurring inhibitors isolated from rice grains (Table I) [11,14]. Since several herbivorous insect pests use cathepsins for protein digestion [16,25–27], such an application of the GST gene fusion system provides a good source of OCs for detailed biochemical studies on their effect toward insect pest proteinases. As the respective specificities of OCI and OCII against cathepsin H and papain were essentially unchanged as compared to the natural inhibitors (Table I), the recombinant inhibitors may also be used to compare their relative efficiency against these proteinases.

Table I

Response of cathepsins B, H, L and papain to recombinant oryzacystatins

Proteinase	Inhibition*	
	OCI	OCII
Cathepsin B	no	no
Cathepsin H	yes (1.13×10^{-6})	yes (2.45×10^{-8})
Cathepsin L	no	no
Papain	yes (3.64×10^{-8})	yes (1.14×10^{-6})

*Values in parentheses correspond to K_i constants (M).

The present study considered the case of CPB, a herbivorous Coleoptera well-known for its remarkable capacity of adaptation against chemical insecticides [28]. Both OCI and OCII were shown to significantly inhibit proteolytic activity in CPB extracts (Fig. 2), and thus appear potentially useful to control this pest. This is the first report indicating the potential of OCII for such an application. For OCI, recent studies showed its potential against several stored grain Coleoptera [12,13]. The present study demonstrates its potential for the control of CPB, a defoliating insect causing considerable losses in North American and East European potato cultures.

Further, the inhibition of CPB digestive proteinases by OCI and OCII was shown to be selective. Roughly, there was a linear relationship between the amount of inhibitor and the percent inhibition of CPB proteinases, until reaching a maximal inhibition value of 20 to 80% for pHs varying from 5.0 to 7.0, respectively (Fig. 2). This pH-dependent inhibition was due to a selective inactivation of CPB cathepsin H by OCs, as demonstrated by the use of cPIs with different specificities (Fig. 3). Such a selective effect could explain partial inhibitions by OCI recently reported for dark mealworm (*Tenebrio obscurus*) and yellow mealworm (*Tenebrio*

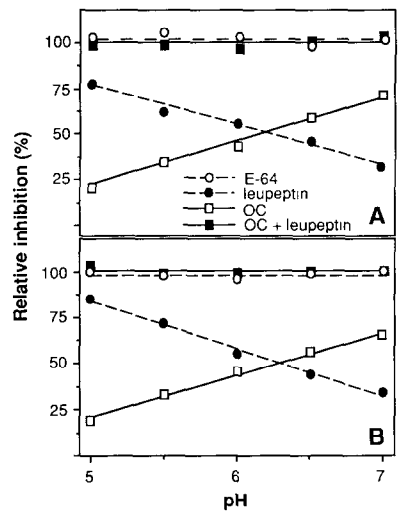

Fig. 3. Inhibition of Colorado potato beetle proteinases by the cysteine proteinase inhibitors E-64 (A,B), leupeptin (A,B), oryzacystatin I (A) and oryzacystatin II (B). The inhibition assays were performed at pHs varying from 5.0 to 7.0 on 3rd instar proteinases. Inhibitor concentrations were 50 µM for E-64, 200 µM for leupeptin, and 50 µg/100 µg midgut protein for OCI and OCII.

molitor) proteinases [13]. It also indicates the importance of having an adequate knowledge of proteolytic systems characterizing the insects to be controlled. For example, the use of OCs for the control of the cowpea seed weevil (*Callosobruchus maculatus*) or the bean bruchid (*Acanthoscelides obtectus*) would not be relevant, since these Coleoptera species principally use cathepsin B for protein digestion [25–27].

In summary, the present study provided evidence that OCI and OCII represent potential growth suppressing agents for the control of the economically important potato pest CPB. The two inhibitors specifically inhibit cathepsin H, which represents an important fraction of the CPB proteinases. OCs could be used either as applied biocontrol agents or as exogenous proteins produced in transgenic potato plants. Studies are currently underway to determine the respective efficacy of OCI and OCII on CPB growth and development by the means of transgenic potato plants.

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