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CHAPTER 2

Characterization of the digestive proteases in the banana weevil gut and the effects of recombinant phytocystatins on early larval growth and development

2.1   Abstract

It is well-documented that insects posses different protease forms used to digest dietary proteins. Therefore, studies to characterize the forms of protease are important to provide the basis for selecting appropriate protease inhibitors likely to be effective in a transgenic approach. In this study the protease activity in the gut of banana weevil was analysed in order to determine the potential of phytocystatins (OC-I and papaya cystatin) for the control of the banana weevil *Cosmopolites sordidus* G. (Coleoptera: Curculionidae). Extracts from complete weevil larvae guts were found to hydrolyse casein at an acidic pH optimum (pH 5.5). Lesser activity was also detected at alkaline pH conditions (pH 8.0). Cathepsin L and B like cysteine proteases were found in the larval gut as shown by hydrolysis of the specific substrates Z-Phe-Arg-MCA and Z-Arg-Arg-MCA, respectively. In addition, activity of trypsin and chymotrypsin-like serine proteases were also detected using the specific substrates Bz-Arg-MCA and N-Suc-Ala-Ala-Pro-Phe-MCA, respectively. OC-I and papaya cystatin produced as a His-tagged fusion protein in *Escherichia coli* and purified by affinity chromatography inhibited cysteine protease activity in the banana weevil gut homogenates by 66.2 and 81.6% and LD50's of 1×10^{-5}ng/ml and 2.1×10^{-5}ng/ml, respectively. A new bioassay was applied to evaluate the effect of OC-I on early growth and development of the larvae. After banana stem disks were vacuum infiltrated with purified OC-I., weight gain per day of larvae was inhibited by 77% at an inhibitor concentration of 0.6mg of cystatin/g fresh weight. This part of the study demonstrated that the banana weevil uses cysteine proteases similar to cathepsin L and B for protein digestion and metabolism in the gut while phytocystatins are potential control agents for banana weevil growth.

2.2 Introduction

Numerous protease inhibitors have been isolated from numerous plants species and there is evidence that they contribute to the natural defense against insect and pathogen attack (Green and Ryan, 1972; Jacinto *et al*., 1998). Several studies have already demonstrated the effectiveness of protease inhibitors for the control of various pests when engineered into transgenic plants. Lecardonnel *et al*. (1999) found increased resistance to the Colorado potato beetle (*Leptinotarsa decemlineata*) by developing transgenic potatoes expressing OC-I. Furthermore, Newell *et al*. (1995) developed sweet potato plants expressing cowpea trypsin inhibitors and found resistance to the West Indian sweet potato weevil (*Euscepes postfasciatus*).

There are generally two major protease classes in the digestive systems of phytophagus insects, either the serine or the cysteine class. Serine protease activity is characteristic of Lepidoptera, Dictyoptera and Hymenoptera while the cysteine class is characteristic of Odoptera and Hemiptera. Initial investigations had concluded that Coleopteran insects mainly use cysteine proteases (Gatehouse *at al*., 1985; Murdock *et al*., 1988). However, from more recent work it appears that a combination of both serine and cysteine proteases is active in this more advanced order (Mochizuki, 1998) suggesting a higher diversity of proteases in these insects.

The objectives of this study were to identify the major classes of proteolytic activity in the gut of banana weevil larvae using *in-vitro* and *in-vivo* assays in order to determine the protease classes present in the weevil. A further objective was to evaluate the potential of OC-I and papaya cystatin to control growth of banana weevil larvae by targeting the cysteine proteases in the weevil gut.

2.3   Materials and methods

2.3.1   Reagents

Azocasein, N- Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Z-Arg-Arg-MCA), Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride (Z-Phe-Arg-MCA), Z-L-arginine-4-methyl-7-coumarinylamide hydrochloride (Z-Arg-MCA), N-Succinyl-Ala-Ala-Pro-Phe7-amido-4-methylcoumarin hydrochloride (N-Suc-Ala-Ala-Pro-Phe-MCA), Benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride (Bz-Arg-MCA), bovine serum albumin (BSA), trans-epoxysucci-nyl-L-leucylamido-(4-guanidino) butane (E-64), gelatin (porcine type A), Triton X-100, phenylmethylsulfonyl fluoride, ethylenediamine tetra acetic acid (EDTA), Phenylmethanesulfonyl fluoride (PMSF), trypsin-chymotrypsin inhibitor from *Glycine max* (Soybean) (SBTi) and aprotinin were purchased from Sigma (Aston Manor, South Africa). Recombinant OC-I and OC-II, corn cystatin-II (CC-II), stefin-A from human (HSA) were a gift from Prof. D. Michaud, who expressed them using the *S*-transferase (GST) gene fusion system (Michaud *et al*., 1994; Brunelle *et al*., 1999).

2.3.2   Insect colony and maintenance

Adult banana weevils were collected from banana growers in Kwazulu Natal Province (South Africa) and maintained in the greenhouse at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. The weevils were kept in 10 liter plastic buckets and provided with fresh banana stem (pseudostem and corm) material to oviposit. After two days, weevils were moved to a different container to allow development of laid eggs. After one week, corms were dissected to collect 3th to 4th instar larvae. These were quickly stored at $–20^{o}C$ until required.

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2.3.3   Gut extractions and protein concentration determination

Frozen larvae were thawed on ice and dissected in cold distilled water under a stereomicroscope to remove whole guts. The guts were then homogenized in liquid nitrogen followed by addition of 0.15M calcium chloride buffer containing 0.1% Triton X-100 at a tissue to buffer ratio of 0.2g/ml of buffer. The mixture was incubated on ice for 30min and then centrifuged at 15,000rpm for 10min. The clear supernatant was collected into fresh tubes and stored at -20°C. For extracts to be used in gelatin SDS-PAGE (see below), guts were homogenized directly in 100µl gelatin-PAGE sample loading buffer (62.5mM Tris-HCl pH 8.0, 2% sucrose, and 0.001% bromophenol blue). The protein concentration of both types of extracts was determined using the Bio-Rad protein assay kit (Bio-Rad, UK), which is based on the Bradford method with bovine serum albumin as the standard.

2.3.4   Determination of pH optima

To determine the pH optima of the crude larvae extracts, protease activity of the extracts was determined using azocasein as a protein substrate as described by Michaud *et al*. (1995). Basically, 50µl (50µg total soluble protein) of a gut extract were mixed with 450µl of assay buffer (0.1M citrate phosphate buffer for pH 4.0; pH 4.5; pH 5.0; pH 5.5; pH 6.0; pH 6.5 and pH 7.0; 0.1M Tris-HCl buffer for pH 7.5; pH 8.0; pH 8.5 and pH 9; 0.1M glycine buffer for pH 9.0, 9.5 and pH 10). All buffers were made to contain 5mM L-cysteine before use. After pre-activating proteases by incubating the mixture for 10min at 37°C, an equal volume of 2% azocasein (in the respective assay buffer) was added and the complete mixture incubated at 37°C for 3hrs. To stop the reaction, 100µl of 10% (w/v) trichloro-acetic acid was added to the mixture and the mixture incubated for 30min at 4°C. Residual azocasein was removed

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by centrifugation at 12000rpm for 5min at 4°C. To 1.0ml of the supernatant, 1.0ml of 1N NaOH was added to precipitate the hydrolysed azocasein and finally the absorbance of this solution was determined at 440nm in a spectro-photometer. At this wavelength, one unit of protease activity is defined to be the amount of enzyme required to produce an absorbance change of 1.0 in a 1cm cuvette under the conditions of the assay (Sarath, 1989). Reactions were performed in triplicate on a micro-titre plate.

2.3.5   Fluorometric assay

Protease specific proteolytic activity and inhibition by specific inhibitors were investigated using the substrates Z-Arg-Arg-MCA (specific to cathepsin B), Z-Phe-Arg-MCA (specific to cathepsin L), Z-Arg-MCA (specific to cathepsin H), Bz-Arg-MCA (specific to trypsin) and N-Suc-Ala-Ala-Pro-Phe-MCA (specific to chymotrypsin). These are highly sensitive fluorometric substrates. When hydrolyzed by their specific proteases, bound α-amino 4-methylcoumarin (MCA) is released, which is highly florescent and MCA release is determined using fluorescence spectro-photometry.

Hydrolysis of the specific substrates by the gut extract was monitored using hydrolysis progress curves as described by Salvesen and Nagase (1989). For detection of cathepsin B, L and H like activity, reaction mixtures contained 10μl (10μg total soluble protein) of the gut extract, 1μl (1%) substrate solution in DMSO dissolved in 89μl reaction buffer; 0.1M citrate phosphate buffer pH 6.0 with 5mM L-cysteine freshly added for cysteine like activity or 0.1M Tris-HCL pH 8.0 for trypsin and chymotrypsion like activity. Hydrolysis was monitored at room temperature using a

spectro-fluorometer (BMG FluoStar Galaxy) with excitation and emission at 360nm and 450nm, respectively. Reaction rates represented by the slope of the curve were recorded as Fluoresence Units (FU) per unit time. All reactions were performed in triplicate.

Inhibitors for the different protease classes were used to evaluate inhibition of their activity. For that, 1μl of a 1% inhibitor solution (E-64, OCI, OCII, CCII, HSA, STBi, aprotinin and PMSF) prepared in the same reaction buffer was introduced into the protease reaction monitored in the spectro-fluorometer. The reactions were briefly mixed and detection of protease reaction continued until a steady rate was reached. Slope values were determined before addition and after addition of the inhibitor.

2.3.6   Gelatin SDS-polyacrylamide gel electrophoresis

Gelatin SDS-polyacrylamide gel electrophoresis, as described by Michaud (1998) was carried out to quantitatively identify protease activity in gut extracts by visually analyzing gel-separated proteases. Proteins in the gut extracts were separated on a 15% SDS-PAGE which had been co-polymerized with 0.1% gelatin as a protease substrate. After electrophoresis at 4°C and 100V, the gel was incubated in 2.5% Triton X-100 for 30min at room temperature to re-nature the proteases. The gel was then incubated in a proteolysis buffer (0.1M citrate phosphate buffer pH 6.0 and 10mM L-cysteine) at 37°C for 3hrs for protease action. The gel was subsequently transferred to a gel staining solution (25% isopropanol, 10% acetic acid and 0.1% coomassie blue). Protease activity was visualized as clear bands on a blue background. To test inhibition, 5μl of a 1% inhibitor solution of selected proteinacious inhibitors

(oryzacystatin, papaya cystatin, SBTi, aprotinin and EDTA) were pre-incubated with 5µl of extracts for 15min at 37°C before loading 10µl of the reaction mixture.

2.3.7 Cloning of OC-I and PC genes

The strategy followed for cloning the coding sequences of OC-I and papaya cystatin (PC) in frame for protein expression in *E. coli* is outlined in Figure 2.1. The vector system p*QE30, 31* and *32* from the QIAexpressionist kit (Qiagen, Germany) was used. These vectors allow tagging the cloned coding sequence to a 6-histidine tag and purification by nickel chelation chromatography.

Coding sequences of OC-I and PC were excised from the cloning vectors p*AOCI-3* and p*BlCYS1* using the restriction enzymes *EcoR*I and *Pst*I. The *EcoR*I/*Pst*I fragments were then first cloned into the *EcoR*I/*Pst*I site of p*BlueScript* (Stratagene, USA) and then as a *BamH*I/*Kpn*I fragment from p*BlueScript* into the vector p*QE31* to achieve in-frame ligation. This sub-cloning procedure created the vectors p*QOC-I* and p*QPC*, which were transformed into *E. coli* cells (strain JM109) and stored in 10% glycerol stocks at –80°C. In a final step, vectors p*QOC-I* and p*QPC* were transferred into competent *E. coli* cells of strain M15 for expression according to the QIAexpressionist kit users manual (Qiagen, Germany).
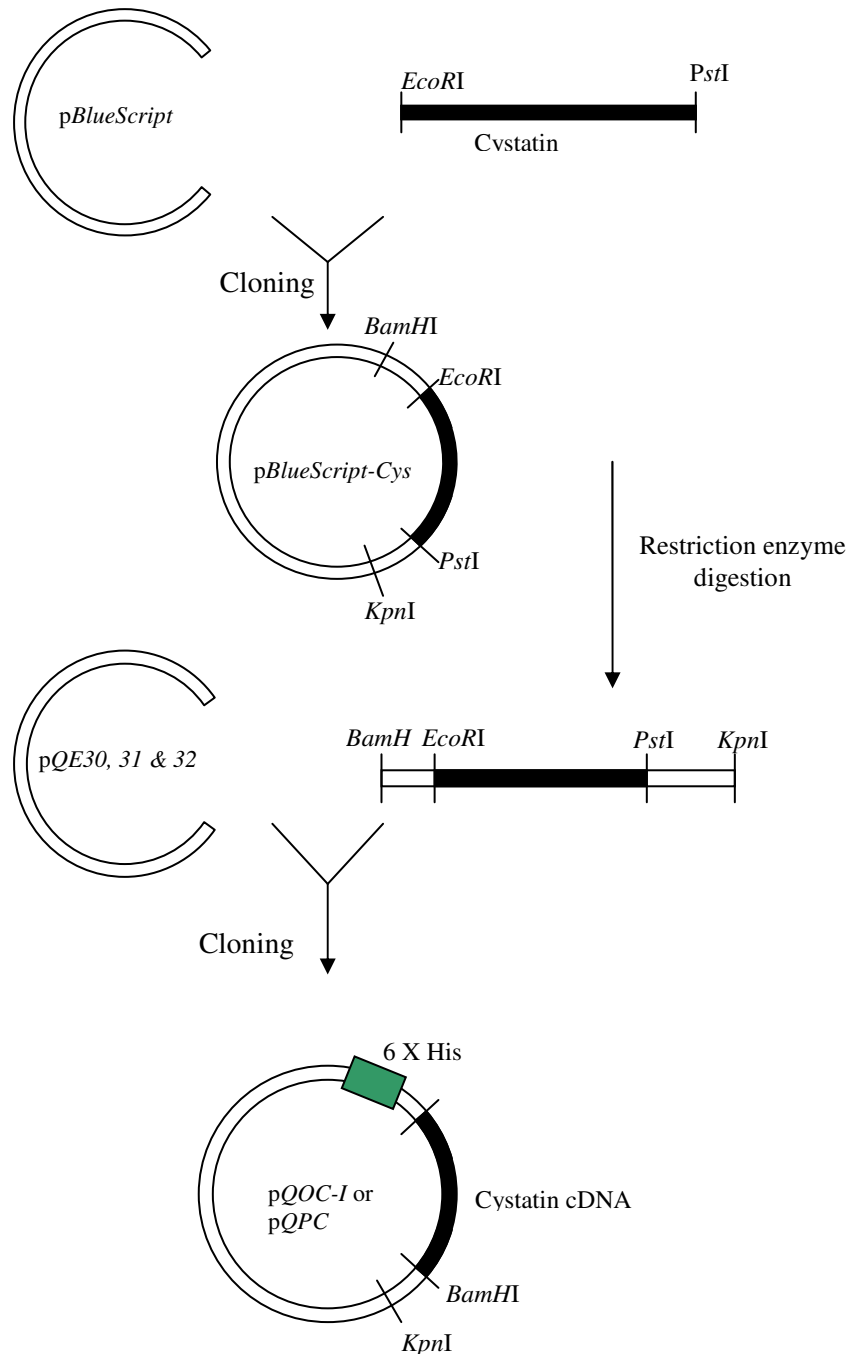
Figure 2.1 Schematic diagram of the construction of expression vectors p*QOC-I* and p*QPC* used in the study to express OC-I and PC in *E. coli*, respectively.

2.3.8   Protein expression and purification

Luria-Bertani (LB) medium (5ml) consisting antibiotics (100μg/ml kanamycin and 25μg/ml ampicillin) was inoculated with a single bacterial colony of M15 cells containing either p*QOC-I* or p*QPC-I* and grown overnight at 37°C under shaking at 210rpm. Pre-warmed LB medium (100ml) with antibiotics (100μg/ml kanamycin and 25μg/ml ampicillin) in a 250ml conical flask was inoculated with 5ml of the overnight culture and incubated at 37°C under shaking at 210rpm for 1hr. Isopropypyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1mM to induce protein expression and bacterial cell growth in the presence of IPTG continued for another 4hr for PC expression or for 12hr for OC-I expression. Cells were finally harvested by centrifugation at 13000rpm in an Eppendorf 5414 Bench top Centrifuge at 4°C for 10min and then stored frozen at -20°C until purification.

The two fusion proteins were purified according to the standard protocol provided in the QIAexpressionist kit manual (Qiagen, Germany). For that, frozen cell pellets were thawed on ice for 30min, re-suspended in his-tag lysis buffer containing 50mM sodium di-hydrogen phosphate, pH 8.0; 300mM sodium chloride and 10mM imidazole at a rate of 2ml buffer per 1mg of cells and 1mg of lysozyme was added. This was mixed gently and incubated on ice for 1hr. The cell suspension was then sonicated using a Cell Disruptor B-30 sonicator (Branson Sonic Power Co./SmithKline Co.) fitted with a standard micro-tip and set to 20% duty cycle, 2 output control in pulse mode. The cells were sonicated using 10 bursts with 10sec cooling on ice between each burst, taking care not to create much frothing. Lysates obtained were centrifuged at 10,000rpm for 30min at 4°C in an Eppendorf centrifuge and the clear supernatant transferred into fresh Eppendorf tubes to which 800μl of

50% Ni-NTA slurry (Qiagen, Germany) was added. The tubes were shaken at 200rpm for 30min at 4°C after which the cell lysate mixture was poured into a short plastic column (setup with a 2ml syringe and a glass wool plug at the bottom) with a paper bottom cover in place. The cover was removed after the slurry settled and the flow-through was collected. Twice 1ml washing buffer (50mM sodium di-hydrogen phosphate, pH 8.0; 300mM sodium chloride; 50mM imidazole) was carefully poured over the column and the buffer was collected at the bottom. This was followed by pouring slowly four times 500µl elution buffer (50mM sodium di-hydrogen phosphate, pH 8.0; 300mM sodium chloride; 250mM imidazole) over the slurry. The elutions were collected separately in 500µl fractions. Five micro-liters of each fraction (flow-through, washes and elution fractions) were each added to 5.0µl SDS-PAGE sample buffer (6% β-mercaptoethanal, 6% SDS, 0.6% bromophenol blue, 20% glycerol) boiled for 10min and loaded onto a 15% polyacrylamide gel for evaluation of the purification process and detection of the recombinant proteins. The protein concentration of the elution fractions was finally determined using the Bio-Rad protein assay kit (Bio-Rad, U.K), and fractions were stored in aliquots at 4°C until required.

2.3.9   In-vitro assays with recombinant phytocystatins

Assays were carried out using a modified method as described by Abrahamson (1994). For that gut extract samples containing 0, 10, 20, 50, 100 and 150µg/µl soluble protein were placed into micro-centrifuge tubes and diluted to 250µl with 0.1% Tween-20. Proteolysis buffer (125µl) was then added (340mM sodium acetate, 60mM acetic acid, 4mM di-sodium EDTA, pH 5.5) and then 8mM DTT was added shortly before use. The mixture was pre-incubated in the presence or absence of the

inhibitor for 1min in a water bath (37°C) before 125µl of substrate (20µM Z-Phe-Arg-AMC, prepared by diluting a 1mM stock in DMSO) was added. Incubation was continued for exactly 10min after which the reaction was stopped by the addition of 1 ml stopping reagent (10mM sodium monochloroacetate, 30mM sodium acetate, 70mM acetic acid, pH 4.3). The florescence of released AMC was determined with the use of a fluorescence spectrophotometer (Hitachi, Model F-2000) with excitation and emission set at 370nm and 460nm, respectively.

2.3.10 Infiltration of banana stem with phytocystatin

Banana inner stems, which form part of the fruit (bunch) stalk but running in the centre of the pseudo-stem from the bunch to underground stem (corm), were collected fresh from the field. In the laboratory, the stem was cut into 1cm disks and dipped into hot 60°C sorbic acid solution (1%) used as a preservative to prevent rapid oxidation and deterioration of the stem disks. Disks were then wrapped into polythene bags and stored at 4°C. Inhibitor solutions for infiltration were prepared by diluting purified his-tagged OC-I and PC to a 10µg/ml solution and 2ml of this solution was placed into a 5cm diameter petri-dish. As a negative control, elution buffer was used in the same way as the inhibitor solutions. Three 4cm long and 1mm diameter thick plastic rods were placed into the petri-dish. One banana stem disk was placed on the rods to prop the disk just above the bottom of the dish to provide the tissue uniform contact with the solution (Figure 2.7A). The complete set-up was then placed into a vacuum desiccator and the dessicator was attached to a vacuum pump (Savant SC100 SpeedVac equipped with a Savant RT100 refrigerated condensation trap). Vacuum was then applied until bubbling was observed on the surface of the tissue and on the solution. The vacuum was then rapidly removed by unplugging a conveniently placed

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(between the desiccator and the pump) tap plunger. This caused the liquid to be drawn into the tissue rapidly. The tissue was removed and placed onto a paper filter in a clean petri-dish and a newly hatched banana weevil larvae were placed in a small hole made on the disk. The treated disks were stored in the dark at 25°C. After 10 days, when the disks were almost decayed, the larvae were dissected out. They were weighted and their head capsule lengths measured (dorsal inter-ocular plane) under a stereo microscope to determine their instar stage as described by Gold *et al.* (1999a).

2.4   Results

2.4.1   pH optima

The optimal hydrolysis of a general protein substrate, azocasein, by banana weevil larval gut homogenates was found to range from pH 5.5 to pH 7.0 with a peak at pH 6.5. There was also a smaller hydrolysis peak (pH 8.5) indicating the presence of both acidic and alkaline proteases in the weevil larval gut (Figure 2.2). Hydrolysis at pH 6.5 was at least 2.5-fold higher than that at pH 8.5. This suggests that acidic cysteine proteases were more predominant in the gut extracts.

2.4.2   Fluorometric assays

To further elucidate on the nature of cysteine and or serine proteases, activity assays were carried out using specific fluorescent substrates. Reaction rates were monitored by detection of the fluorescent product MCA. Two types of cysteine proteases, cathepsin-L and cathepsin-B, were the predominant cysteine protease types producing reaction rates of >1000FU/sec/μg total protein compared to <400FU/sec/μg total protein from cathepsin-H, trypsin and chymotrypsin (Figure 2.3). Of the serine

proteases, trypsin showed the lowest significant proteolysis at 177FU/sec/µg total
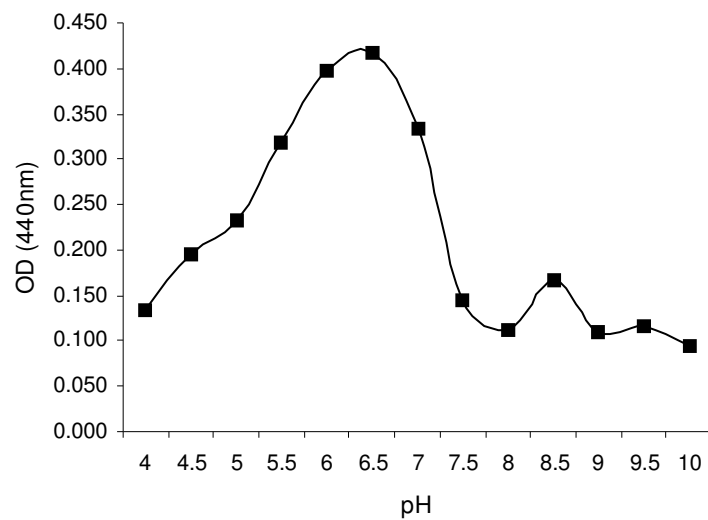
protein (Figure 2.3).

Figure 2.2   Effect of pH on the hydrolysis of azocasein by banana weevil larval gut

proteases. Proteolysis was stopped by the addition of 1.0ml of 1N NaOH

and the OD of the solution determined at 440nm. At this wavelength,

one unit of protease activity is defined to be the amount of enzyme

required to produce an absorbance change of 1.0. Reactions and

measurements were performed at room temperature. Experiment was

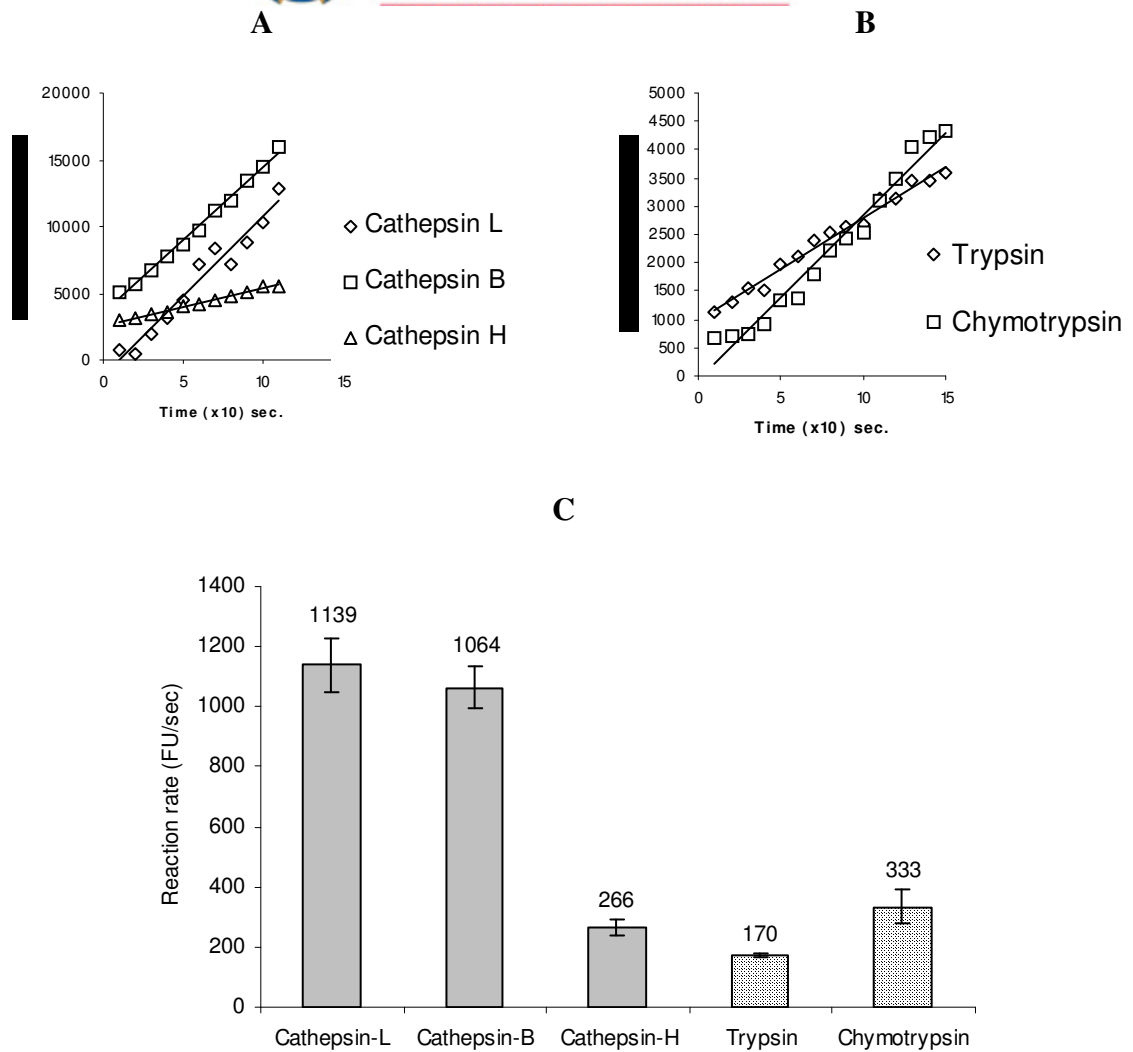repeated twice and values shown are the mean of 3 individual

experiments.

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A   **B**

C

Figure 2.3   (A) Cathepsin B, L and H like activities detected in banana weevil

larval gut extracts. Fluorometric assays were conducted using Z-Arg-

Arg-MCA, Z-Phe-Arg-MCA and Z-Arg-MCA as substrates for

cathepsin B, L and H like activities, respectively at pH 6.0. (B) Trypsin

and chymotrypsin-like activities detected in the same extracts, using

Bz-Arg-MCA and N-Suc-Ala-Ala-Pro-Phe-MCA as substrates

respectively performed at pH 8.0. (C) Maximum activities of all

proteases tested. Data points and graphs shown represent the means of

three replications ±SE.

The effects of selected inhibitors were accessed using a banana weevil gut extract and cysteine and serine protease specific substrates. E-64 was the most potent inhibitor of cathepsin L and B like activity with 96% and 85% inhibition of protease activity, respectively. OC-I was the most potent natural plant cysteine protease inhibitor of cathepsin L and B-like activity with 81% and 80% inhibition of protease activity, respectively (Table 2.1A). The soybean trypsin-chymotrypsin inhibitor was most the potent inhibitor against trypsin and chymotrypsin-like activity with 92% and 98% inhibition, respectively. Aprotinin, a serine protease inhibitor, showed lower inhibition of chymotrypsin-like activity when compared to inhibition of trypsin-like activity (Table 2.1B).

2.4.3   Gelatin SDS-polyacrylamide gel electrophoresis

The use of gelatin-containing PAGE gels offers a visual assessment of the protease profile in a crude extract by separating the proteases into their individual constituents. This provides a more detailed profile of protease activity. Extracts were therefore pre-incubated with selected inhibitors before separation on a 15% SDS-PAGE. Figure 2.3 shows that extracts contained at least five different proteases with different molecular sizes of 22, 25, 30 72 and 170kDa.

Table 2.1 Inhibition of banana weevil gut proteases by (A) cysteine and (B) serine protease inhibitors. Cysteine protease inhibitors tested were E-64, OC-I, OC-II, corn cystatin (CC-II) and human stefin A (HSA). Serine protease inhibitors tested were soybean trypysin and chymotrypsin inhibitor (STBi), aprotinin and phenylmethylsulphonylfluoride (PMSF). Reactions for serine protease inhibition were performed in 100mM Tris-HCl buffer (pH 8.0) at room temperature. Proteolytic activity was measured as a rate of reaction indicated by fluorescence units (FU) produced per second per µg of protein (FU/Sec/µg). Control represents reaction in substrate without addition of an inhibitor. Data represent the mean of three replications ±SE.

A

	Cathpesin L-like		Cathepsin B-like	
	FU/sec/µg	Inhibition (%)	FU/sec/ µg	Inhibition (%)
Control	1278.2 ±131	-	1151.1±59	-
E-64	47.4 ±73	96	175.2±19	85
OCI	238.3 ±59	81	228.3±6	80
OCII	394.1 ±106	69	495.0±10	57
CCII	225.6 ±36	82	306.0±23	73
HSA	294.5 ±30	77	593.7±20	48

B

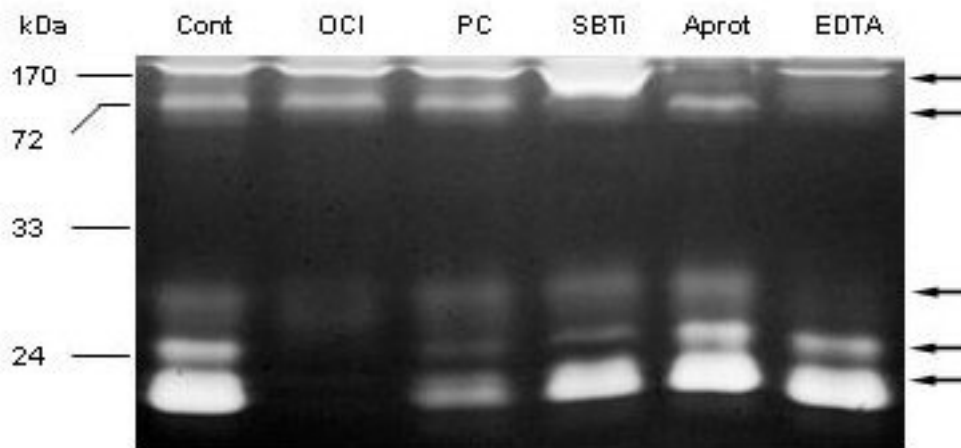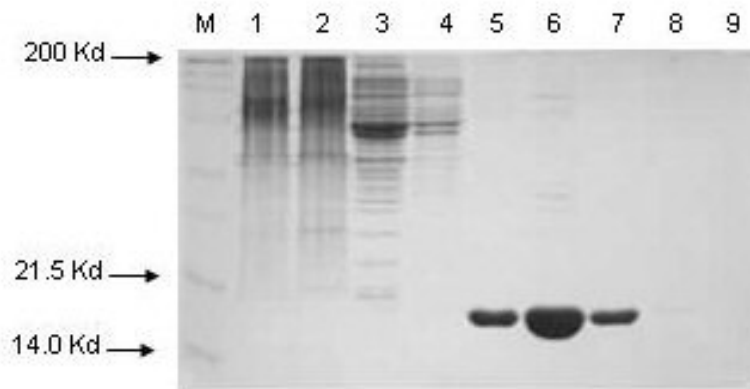|	Trypsin-like		Chymotrypsin-like	
	FU/Sec/µg	Inhibition (%)	FU/Sec/µg	Inhibition (%)
Control	185.0±15	-	297.8±32	-
SBTi	14.5±1	92	7.3±10	98
Aprotinin	18.3±11	90	168.9±15	43
PMSF	108.9±14	41	66.3±6	78

Figure 2.4   The effect of protease inhibitors on the proteolysis activity of banana weevil larval gut proteases revealed by separation in a mildly denaturing 15% SDS-PAGE co-polymerized with gelatin. Protease activity measurement was carried out in a buffer containing 100mM citrate phosphate and 10mM mercaptoethanol, pH 6.0. Visible clear bands indicate proteolysis of gelatin. (Cont) represents activity of crude gut extract (3µl from a 200µg/µl solution); (OC-I) pre incubation with 20µg of OC-I; (PC) pre-incubation with 20µg papaya cystatin; (SBTi) pre-incubation with 1% soybean trypsin-chymotrypsin inhibitor; (Aprot) pre-incubation with 1% aprotinin and (EDTA) pre-incubation with100mM EDTA. Arrows indicate major protease activities.

The most potent inhibitor in this assay was OC-I followed by PC, which had not been used in the previous fluorometric assay. Both cystatins reduced the protease activity profile from 5 to 3 bands including a major activity band at 22kDa (Figure 2.4). The soybean trypsin-chymotrypsin inhibitor (SBTi) inhibited one band at 72kDa while aprotinin inhibited one band at 170kDa. EDTA showed inhibition of one band at 30kDa. This suggests the presence of some metallo-proteases in the gut extract. Figures 2.5A and B show the expression and purification of both PC and OC-I as his-tagged fusion proteins, respectively. Protein bands with the expected size of about 15.0Kd for PC and 18Kd for OC-I were found. However, expression levels of OC-I were much lower than of PC. This required the OC-I cultures to be incubated in the presence of IPTG for a longer time period (12hr) when compared to PC (4hr). Reduced growth might be due to OC-I toxicity in *E. coli*. Purified his-tagged PC and OC-I reduced cysteine protease (cathepsin L like) activity of weevil larval gut extracts by 66.2 and 81.6%, respectively (Figure 2.6). The calculated LD50 of inhibition by PC was 2.1×10^{-5}ng/ml and for OC-I 0.1×10^{-5}ng/ml.
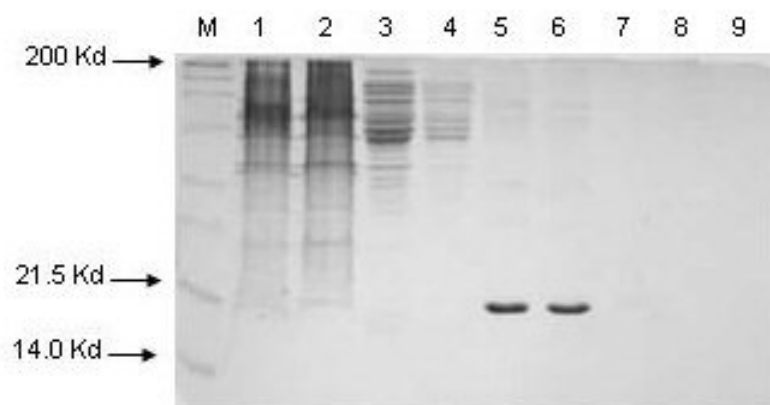
A

B

Figure 2.5   SDS-PAGE of (A) PC and (B) OC-I at different purification steps. Lane 1 represents a broad range protein marker (BioRad); lane 2 non-induced and lane 3 IPTG-induced proteins expressed in *E. coli* cells; lane 4 flow-through from Ni-NTA column; lane 5 wash-through from column and lanes 6-9 are four consecutive elutions from column used for purification. Slower migration speeds of recombinant cystatins may be due to presence of his-tags and high concentration of imidazol in the elution buffer.
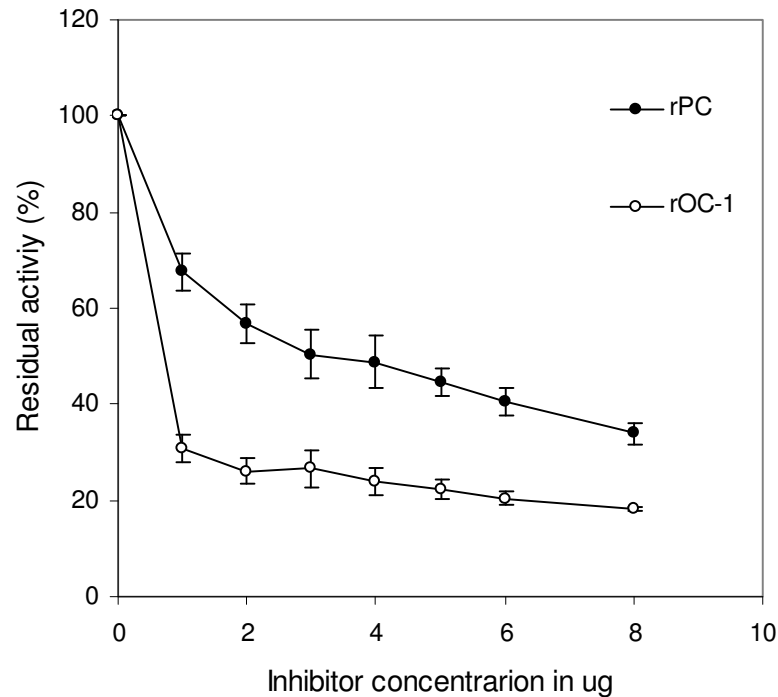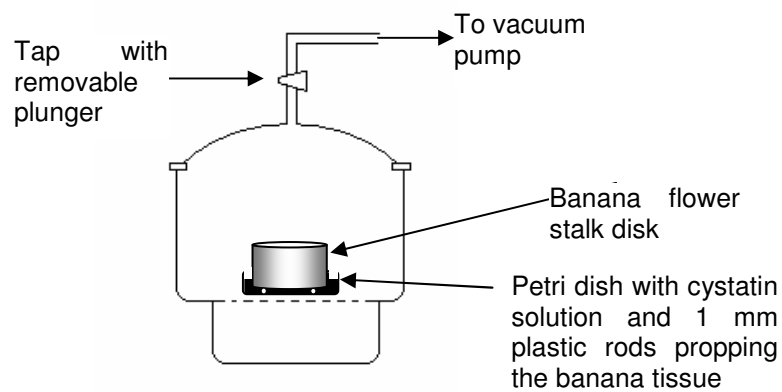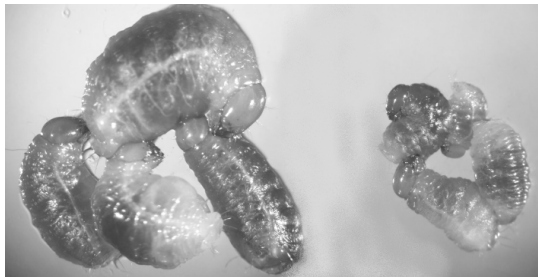
Figure 2.6   The effect of recombinant cysteine protease inhibitors rOC-I and rPC on

the cysteine protease activity of banana weevil mid-gut extracts using Z-

phe-arg-AMC as substrate.

When larvae fed on banana disks infiltrated with both purified phytocystatins, their

development was significantly reduced. Early larval developmental rate was reduced

for both phytocystatins. Body weight gain was 0.25mg/day for OC-I and 0.35mg/day

PC compared to 1.1mg body weight gain per day in the control larvae (Figure 4.4 C).

This represents a reduction in development of 77% for OC-I and 68% for PC at a

concentration of 0.6mg/g fresh weight of infiltrated stem disk after re-extraction

(Figures 2.7 B and C). However, there was no significant difference in weight gain /

day between OC-I and PC-treated larvae ($p > 0.05$).
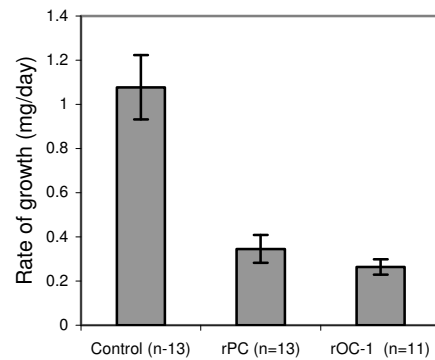
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A

B

C

Figure 2.7   (A) Illustration of the apparatus used to vacuum infiltrate banana flower stalk disks with cystatin solution. (B) Larvae on the left after developing on cystatin-free (control) disks for 10 days, while larvae on the right developed in cystatin treated disks over the same period. (C) The growth rate of larvae that were reared on banana stem disks vacuum-infiltrated with a 100ug/ml (to give a final 0.6mg of recombinant protein per disk) solution of recombinant rOC-I and rPC. Values represent means of 39, 38 and 22 replicates for control, rPC and rOC-I, respectively

2.5   Discussion

Many efforts to develop insect resistance in a plant via the expression of protease inhibitors have resulted only in a few successes (Winterer, 2002). Several studies have shown that many insects have more than one protease forms and their activity in gut protein digestion and metabolism is influenced by several factors (Gatehouse *et al*., 1993), such as gut pH (Michaud *et al*., 1993), larval stage (Orr *et al*., 1994) and the quantity and quality of the protein diet (Burgess *et al*., 1991). This study has provided first evidence that the banana weevil larval, the most destructive stage of the pest's life cycle, expresses a variety of proteases, including cysteine proteases, in its gut. This protease can be blocked by phytocystatins. In contrast, this study showed that serine and metallo-proteases very likely play a less prominent role in protein digestion by larvae. Any strategy to use protease inhibitors to target the banana weevil needs therefore to consider that the weevil possesses more than one protease class. In this study strong evidence has been further provided that both OC-I and PC are able to control the development of the banana weevil by blocking gut cysteine proteases. Significant reduction in the body weight and thus rate of growth of the larvae due to inhibitor action contributed to the larvae underfeeding and interference with protein digestion and metabolism. However, it has always to be considered that even if serine types seemingly play a less significant role in the gut profile when compared to cysteine proteases, weevils might switch to serine type proteases to overcome the presence of cystatins in the diet.

Since there are no transformed banana plants available yet to express an endogenous phytocystatin, the developed vacuum infiltration assay was a very useful and simple tool to access the effects of phytocystatins on growth and development of the banana

weevil larvae. There is even further potential to scale up this infiltration assay so that the assay period is extended to the pupa and adult stage of the larvae. Although this experiment demonstrated the potential of phytocystatins to block weevil development, the infiltration experiments were carried out with a relatively high inhibitor concentration of 0.6mg/g of fresh weight after re-extracting the recombinant proteins from infiltrated stem disks. Such concentrations are difficult to achieve in transgenic approaches to effectively extenuate pest insect gut proteases. However, this study used a single dose of the phytocystatin whose effect may have deteriorated with time of culture. Further experiments have to demonstrate if a lower phytocystatin but continuously expressed in a transgenic plant might result in a similar growth inhibition.

Overall, this study confirmed that icysteine proteases are important protein digestive enzymes in the gut of the banana weevil. The two phytocystatins studied are able to significantly reduce developmental success of the banana weevil larvae. The newly developed bioassay system has been found to be a useful tool for testing bioactive compounds on banana weevil larvae growth and development. Finally, this study provided also first evidence that a transgenic strategy to use protease inhibitors expressed in banana in the control of the banana weevil is plausible. Due to the presence of both cysteine and serine protease in the gut, this study also suggests that simultaneous expression of cysteine and serine protease inhibitors might be a strategy to prevent larvae growth and development.