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CmCatD, a cathepsin D-like protease has a potential role in insect defense against a phytocystatin

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ABSTRACT

When fed on a diet containing a proteinaceous cysteine protease inhibitor from soybean (scN), cowpea bruchid larvae enhance their overall digestive capacity to counter the inhibitory effect. Elevated proteolytic activity is attributed not only to the major digestive cysteine proteases (CmCPs), but also to aspartic proteases, a minor midgut protease component. In this study, we isolated a *CmCatD* cDNA from cowpea bruchid midgut that shares substantial sequence similarity with cathepsin D-like aspartic proteases of other organisms. Its transcript profile was developmentally regulated and subject to alteration by dietary scN. *CmCatD* transcripts were more abundant in scN-fed 3rd and 4th instar midguts than in control. The bacterially expressed recombinant CmCatD proprotein was capable of autoprocessing under acidic conditions, and mature CmCatD also exhibited pH-dependent proteolytic activity which was inhibited specifically by pepstatin A, indicative of its aspartic protease nature. CmCatD trans-activated CmCPs and vice versa, suggesting a cooperation between the minor midgut CmCatD and major digestive CmCPs. Further, CmCatD was able to degrade scN after extensive incubation. This activity partially restored CmCP proteolytic activity otherwise inhibited by scN. Thus CmCatD could facilitate insects' coping with the challenge of dietary scN by exerting its scN-insensitive and scN-degrading activity, freeing cysteine proteases for food degradation. Taken together, cowpea bruchids coordinate the functionality of the two classes of digestive proteases to fend off the negative effect of scN, and fulfill their nutrient requirements.

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1. Introduction

In response to insect herbivory, plants have evolved defense mechanisms including anti-nutritional proteins such as protease inhibitors to debilitate insect proteolysis. Although it held great promise for environmentally friendly insect control, overexpression of plant defensive protease inhibitors in transgenic crops has been largely unsuccessful. Resistance to protease inhibitors appears to be a common phenomenon (Jongsma et al., 1995; Jongsma and Bolter, 1997; De Leo et al., 1998; Cloutier et al., 2000; Mazumdar-Leighton and Broadway, 2001; Zhu-Salzman et al., 2003; Gruden et al., 2004; Yang et al., 2009). Mechanistic studies of insect resistance to plant defense have led to an appreciation of the remarkable diversity and plasticity of their digestive proteases. Insect digestive proteases, responsible for breakdown of food proteins, are characterized into serine-, cysteine-, aspartic- and metallo-proteases (Terra and Ferreira, 1994). A specific insect species often possesses multiple digestive proteases in their alimentary tract, belonging to different or the same mechanistic groups, although it typically utilizes one major type to serve the digestive role (Silva and Xavier-Filho, 1991; Oppert et al., 1993; Brunelle et al., 1999; Liu et al., 2004). It is postulated that having multiple digestive enzymes could be a functional overlap to ensure nutritional protein degradation. A less apparent function of insect digestive enzymes is the role they play in coping with plant defense proteins.

We have previously used the cowpea bruchid (*Callosobruchus maculatus*) as a model to study insect counter-defense. Larvae, the developmental stage that is associated with food intake and digestion, feed on stored cowpea and other grain legumes, causing serious damage. Like many other coleopterans, the cowpea bruchid has a midgut pH of *ca.* 5.5 and uses cathepsin L-like cysteine proteases, referred to as CmCPs in this study, as its major digestive enzymes (Zhu-Salzman and Salzman, 2001). Aspartic protease activity has also been detected in the bruchid gut extract, accounting for approximately 10% of the total gut proteolytic activity under normal growth conditions (Kitch and Murdock, 1986; Silva and Xavier-Filho, 1991; Zhu-Salzman et al., 2003). Inhibition of the minor digestive proteases in the bruchid digestive

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tract by incorporating the aspartic protease-specific inhibitor pepstatin A in the diet significantly prolonged the developmental time of this insect (Amirhusin et al., 2007).

Cathepsin D is one of the best studied aspartic proteases in vertebrates and arthropods, and is responsible for intracellular and extracellular protein digestion through limited proteolysis (Lenarcic et al., 1991; Silva and Xavier-Filho, 1991; Terra and Ferreira, 1994; BlancoLabra et al., 1996; Brunelle et al., 1999; Tcherepanova et al., 2000; Lkhider et al., 2004; Boldbaatar et al., 2006; Erdmann et al., 2008). These proteases have acidic pH optima ranging from pH 3 to 5, and rely on two aspartate residues for catalysis. Cathepsin D is involved in vitellogenin production and degradation in mosquito (Cho and Raikhel, 1992), while in medfly (*Ceratitis capitata*) its activity coincides with fat body histolysis (Rabossi et al., 2004). In silkworm (*Bombyx mori*), its involvement in programmed cell death is the key to the larval–pupal transformation (Gui et al., 2006). Cathepsin D enzymes also conduct blood meal digestion in ectoparasitic mites and ticks (Boldbaatar et al., 2006). Cathepsin D is secreted into the midgut lumen of many coleopterans where cysteine proteases are often the major digestive enzymes (Silva and Xavier-Filho, 1991; BlancoLabra et al., 1996; Brunelle et al., 1999).

When cowpea bruchid larvae were reared on a diet containing a sustained, sublethal dose of soybean cystatin scN, the midgut protease activity profile was dramatically remodeled (Zhu-Salzman et al., 2003). While differentially inducing existing scN-sensitive CmCPs, this insect also activated scN-insensitive and scN-degrading proteolytic activity (Zhu-Salzman et al., 2003; Ahn et al., 2004). Interestingly, a cathepsin D-like cDNA was identified as an scN-induced gene at the 4th instar stage in a large-scale cDNA microarray (Chi et al., 2009). This is consistent with our previous observation in which scN-fed cowpea bruchids had higher aspartic protease activity in the gut extract than that of control insects (Zhu-Salzman et al., 2003). In addition, bioassays that combined pepstatin A and scN exhibited a synergistic anti-insect effect (Amirhusin et al., 2007). These results suggested that the impact of this normally minor digestive enzyme could become significant when the major digestive CmCP enzymes are inhibited. Quantitative and qualitative modulation of the digestive protease complement presumably mediated the recovery of normal feeding and growth at later larval stages.

In this study, we explore the potential accessory function of a midgut aspartic protease in insect adaptive response to dietary scN. The results add a new dimension to cathepsin D functionality and support the concept that insect gut proteases act in a coordinated manner to fulfill roles in nutrient requirement for development and protecting insects against toxic effects of anti-nutritional factors.

2. Materials and methods

2.1. Cloning of the full-length of CmCatD from cowpea bruchid midgut

Midguts from the 4th instar larvae reared on cowpea seeds were dissected following the procedure of Kitch and Murdock (1986). Total RNA was extracted using a TRIzol-based method (Invitrogen, Carlsbad, CA), followed by reverse transcription using SuperScript II Reverse Transcriptase (Invitrogen). A partial *cathepsin D*-like EST clone, obtained previously from a microarray project aimed at identification of scN-regulated midgut genes (Chi et al., 2009), was used to design the gene-specific primer for 5′-RACE PCR. The 5′ cDNA end was amplified by PCR (94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min for 35 cycles) using the BD SMART RACE cDNA Amplification kit (BD Biosciences Clontech, Palo Alto, CA) with BD SMART II A oligonucleotide and antisense gene-specific primer (5′-GGTCCATAATATTGAGCATCCAGG-3′). The PCR fragment was subcloned into the pCRII vector (Invitrogen) and sequenced. The full-length cDNA was then obtained by RT-PCR (94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min for 35 cycles) using the following primers: sense 5′-GGTCATTATTATTAATTTATGTTGTGT-3′; antisense 5′-AAACTGAACATCATTTATTAACTAAAA-3′. The PCR fragment was then subcloned into pCRII, and the sequence was confirmed.

2.2. Quantitative RT-PCR

The mRNA level of *CmCatD* was assessed during larval development under dietary scN challenge by real time RT-PCR using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Bruchids were reared on diet containing 0.2% scN as well as scN-free control diet, respectively, and their midguts were collected at 2nd, 3rd and 4th instar stages. Total RNA was extracted from these tissues and reverse transcribed as described above. Gene-specific primers resulting in a 152 bp amplicon were as follows: sense 5′-CACTGATCATGTGTCTTTTGG TGG-3′ and antisense 5′-ACTGGGGGTACTCCATCTACAGC-3′. PCR reactions (47 cycles of at 95 °C for 15 s and at 60 °C for 1 min following an initial incubation at 50 °C for 2 min and 95 °C for 10 min) were performed in SYBR Green Master Mix (Applied Biosystems). PCR amplification of a 12S rRNA fragment (GenBank accession number AF004131) of all samples served for normalization of input cDNA between samples. No template control using untranscribed RNA confirmed that no interfering PCR product derived from genomic DNA were present. All reactions were done in triplicate. The mean induction fold was calculated according to Zhu-Salzman et al. (2003) and plotted using KALEIDA-GRAPH (Synergy Software, Reading, PA). A one-way ANOVA test was used to analyze the mean fold induction during larval development, and Fisher's LSD test ($P = 0.01$) was used for mean separation (SPSS for Windows ver.12.0.1).

2.3. Expression and purification of proCmCatD

The cDNA fragment encoding the proprotein (*proCmCatD*) was PCR amplified (94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, 35 cycles) with the following primers: sense 5′-GTCCTGCATATG-GAATTTCATAGGATACCTTTA-3′; antisense 5′-TTGATCTCGAGT-TAAACAGCTTCTGCAAA-3′. Restriction sites NdeI and XhoI (underlined) were introduced into the primers for directional subcloning. After restriction digestion, the fragment was cloned into pET-28a(+) expression vector (Novagen, Madison, WA), and the construct was subjected to DNA sequencing.

Once correct DNA sequence was confirmed, the construct was transferred to *E. coli* expression host strain BL21 (DE3) (Novagen). Cells were grown until OD_{600} reached between 0.4 and 1.0. The production of recombinant proCmCatD fused with hexa-His tag was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 1.0 mM) overnight at 37 °C. The insoluble recombinant protein was purified as described by Holzinger et al. (1996) with modification. The cell pellet from 500 ml of bacterial culture was lysed in 50 ml of Buffer 1 (6 M guanidine, 0.02 M Tris–HCl, pH 7.9, 0.5 M NaCl), and sonicated (Model 250 Sonifier, Branson, Danbury, CT) followed by incubation on ice for 1 h. After centrifugation at 4 °C, 39,000 × *g* for 30 min, the supernatant containing the recombinant protein was applied to a column containing 5 ml of Ni^{2+}-chelating sepharose (GE Healthcare, Piscataway, NJ) pre-equilibrated with Buffer 1. The column was washed with 65 ml of Buffer 2 (6 M urea, 0.02 M Tris–HCl, pH 7.9, 0.5 M NaCl, 0.02 M imidazole), and the recombinant protein was then renatured by addition of 65 ml of Buffer 3 (0.02 M Tris–HCl, pH 7.9, 0.15 M NaCl) and eluted in 30 ml of Buffer 4 (0.02 M Tris–HCl, pH 7.9, 0.15 M NaCl, 0.3 M imidazole). The purified recombinant protein fused

with His-tag at its N-terminus was concentrated by adding ammonium sulfate to 80% concentration (Englard and Seifter, 1990) and analyzed on 12.5% SDS-PAGE or 15% tricine SDS-PAGE (Schagger and Vonjagow, 1987).

2.4. In vitro propeptide processing of recombinant proCmCatD

To examine autolytic processing, the purified recombinant proCmCatD (1.5 μg) was incubated at 37 °C for 1 h at various acidic pH conditions, buffered by 100 mM citric acid (pH 3.0 and 3.5) and 100 mM sodium acetate (pH 4.0–6.0). In addition, proCmCatD (1.5 μg) was also pre-incubated with pepstatin A (1 μg) prior to autolytic maturation to evaluate its effect on autoprocessing. Preincubation was performed at room temperature for 20 min, at pHs ranging from 3.0 to 5.0.

To evaluate potential trans-processing of proCmCatD by the bruchid major digestive enzymes under physiological pH, bacterially expressed recombinant proCmCPB1 (1.5 μg) was purified and autoprocessed as described previously (Ahn et al., 2004), followed by addition of proCmCatD (1.5 μg) and a further incubation in 100 mM sodium acetate (pH 5.0–6.0) at 37 °C for 1 h. All samples were examined by 12.5% SDS-PAGE.

To determine if mature CmCatD is able to trans-process bruchid digestive CmCPs, 3 μg of recombinant proCmCPA16, a CmCP isoform incapable of autoprocessing (Ahn et al., 2004), was incubated with autoprocessed CmCatD (from 1 μg proprotein) at 37 °C for 0 and 1 h in 100 mM sodium acetate, pH 5.0, respectively. The proteins were subjected to 12.5% SDS-PAGE and immunoblot analysis. The primary antibody (1:500 dilution) was polyclonal rabbit anti-recombinant CmCPA9 antibody (Ahn et al., 2004), and the secondary antibody (1:10,000 dilution) was goat anti-rabbit IgG conjugated with horseradish peroxidase (Kirkegaard Perry Laboratories). Antigen–antibody complexes were detected using Amersham ECL Plus Western Blotting Detection System (GE Healthcare).

2.5. pH optimum and specific inhibition of CmCatD enzymatic activity

A hemoglobin hydrolytic assay was used to evaluate proteolytic activity of CmCatD as described by Barrett (1970) with modifications. This substrate was denatured in 100 mM formic acid (pH 3.5) at the concentration of 2.5%. Subsequently, autoprocessed CmCatD (at pH 4.0) was added. In each reaction, mature CmCatD derived from 3 μg proprotein was combined with 50 μl acid-denatured hemoglobin. Total reaction volume was 800 μl. To determine the pH effect, reactions were conducted in solutions varying in pH (buffered as described above). After 30 min proteolysis at 37 °C, 500 μl of 10% trichloroacetic acid was added, incubated for 10 min at 37 °C, and the residual hemoglobin removed by centrifugation at 15,000 × *g* for 10 min. The supernatant was transferred to a cuvette, and the absorbance at 280 nm was measured using a GENESYS 10 UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The blank was prepared using the same procedure but with zero proteolytic reaction time. All reactions were performed in triplicate. Enzymatic activity was plotted using KALEIDA-GRAPH (Synergy Software). A one-way ANOVA test was used to analyze the absorbance data, and Fisher's LSD test ($P = 0.01$) was used for mean separation (SPSS for Windows ver.12.0.1).

To determine the proteolytic specificity of CmCatD, the processed CmCatD (from 3 μg proCmCatD) was pre-incubated with either water, the aspartic protease inhibitor pepstatin A (at a final reaction concentration of 1.9 μM), the cysteine protease inhibitor trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E-64, 3.5 μM), the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF, 1 mM) or the serine and cysteine protease inhibitor leupeptin (10 μM) for 20 min at room temperature. Mixtures were continuously incubated with 50 μl of 2.5% acid-denatured hemoglobin in 800 μl 0.1 M formic acid, pH 3.5 for 30 min at 37 °C. The proteolytic activity was plotted as previously described, and the reactions were performed in triplicate. A one-way ANOVA test was used to analyze the absorbance data, and Fisher's LSD test ($P = 0.01$) was used for mean separation (SPSS for Windows ver.12.0.1).

2.6. scN hydrolysis by CmCatD

To determine whether CmCatD contributes to scN-degrading activity, autoprocessed CmCatD (derived from 7.5 μg proCmCatD) was incubated, in the presence and absence of pepstatin A (2 μg) respectively, with recombinant scN purified following Zhu-Salzman et al. (2003). Reactions (buffered by 100 mM sodium acetate pH 4.0) were conducted at 37 °C for 0, 6, or 16 h, followed by 15% tricine SDS-PAGE.

The scN-degrading activity of CmCatD may facilitate the proteolysis of CmCPs. To test this, self-matured CmCPA9 (derived from 2 μg proCmCPA9) as described previously (Ahn et al., 2004) was incubated with scN (1 μg), in the presence and absence of the processed CmCatD (from 3 μg proCmCatD) for 0, 6 or 16 h at 37 °C. Such protein quantities equate to a 1:1:1 molar ratio. The proteolytic activity of CmCPA9 on a synthetic chromogenic substrate, benzyloxycarbonyl-L-phenylalanyl-L-arginine-*p*-nitroanilide (Z-Phe-Arg-pNA) (Bachem, King of Prussia, PA) was compared. CmCPA9 and CmCatD alone were used as experimental controls. Briefly, 30 μl of 10 mM Z-Phe-Arg-pNA pre-equilibrated to 37 °C in 100 mM sodium acetate, pH 5.0, 1 mM EDTA, 2.5 mM DTT was incubated with the above mixtures for 20 min at 37 °C. The absorbance was then measured at 410 nm using a GENESYS 10 UV spectrophotometer. Absorbance from the sample with zero time proteolytic incubation was used as a blank. The proteolytic activity was plotted as previously described, and the experiments were performed in triplicate. One unit of protease activity was defined as the amount of mature CmCPA9 required to produce an absorbance change of 0.01 per min in 1-cm cuvette at 37 °C. A one-way ANOVA test was used to analyze the proteolytic activity data, and Fisher's LSD test ($P = 0.01$) was used for mean separation (SPSS for Windows ver.12.0.1).

3. Results

3.1. Alteration of developmentally regulated CmCatD by scN

Previous observations of enhanced midgut aspartic protease activity in cowpea bruchid larvae fed on scN-containing diet led to the hypothesis that this minor digestive enzyme plays a role in the insect coping with scN (Zhu-Salzman et al., 2003). In a separate effort, a partial cathepsin D-like cDNA clone was identified by microarray due to its higher abundance in scN-fed insect midgut than in control insects (Chi et al., 2009). To determine the molecular basis of the cowpea bruchid midgut aspartic protease activity, we cloned the full-length *CmCatD* cDNA. The 1503 cDNA clone (GenBank accession number FJ360435) contains an open reading frame of 1170 bp that encodes a protein of 389 amino acid residues (Fig. 1). Sequence alignment showed a high degree of amino acid similarity with other cathepsin D-like members, such as those from the red flour beetle *Tribolium castaneum* (75.4%, XM_961424), mosquito *Aedes aegypti* (69.3%) (Cho and Raikhel, 1992), silkworm *B. mori* (63.4%) (Gui et al., 2006) and human (58.5%) (Faust et al., 1985). The characteristic two Asp and six Cys residues in cathepsin D were conserved in CmCatD as well.

To profile *CmCatD* expression during larval development in the presence and absence of scN, guts of 2nd, 3rd and 4th instars fed on control and scN-containing diets respectively were collected for

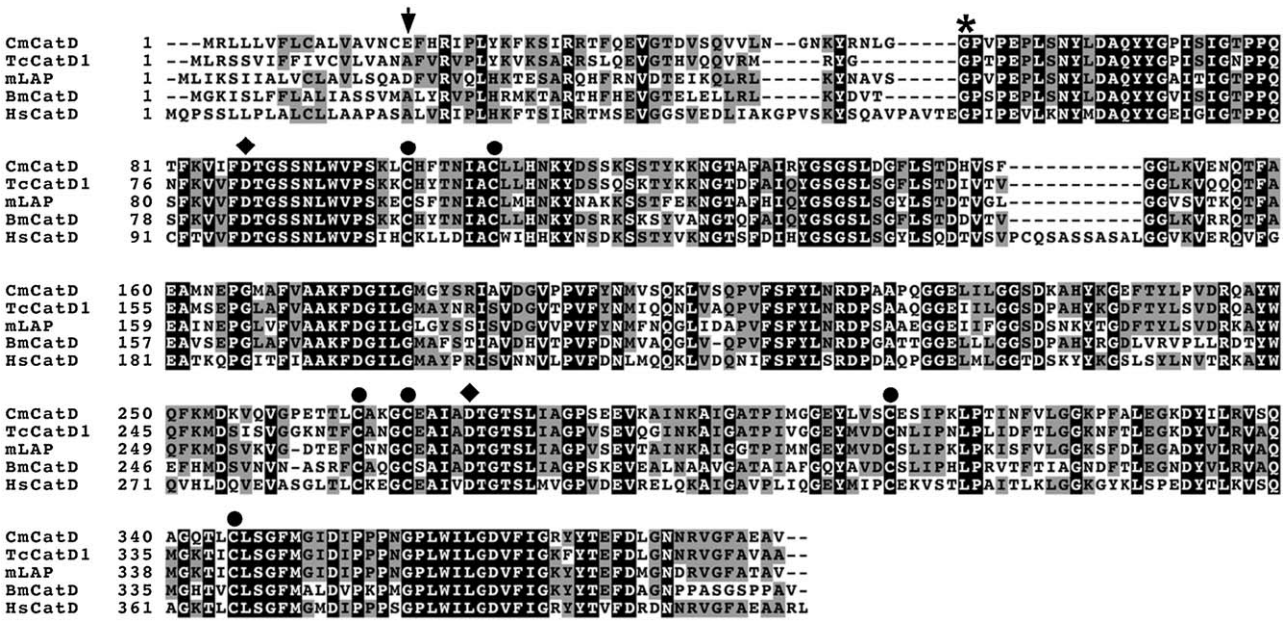

Fig. 1. CmCatD (GenBank accession FJ360435) shares high protein sequence similarity with aspartic protease members from red flour beetle TcCatD1, mosquito mLAP (M95187), silkworm BmCatD (AY297160), and human HsCatD (M11233). Identical residues are shaded dark grey and conserved residues shaded light grey. The dashes represent missing amino acid residues. The catalytic residues are marked with diamonds, and six invariant cysteine residues that form disulfide bridges are indicated with closed circles. The arrowhead refers to the beginning of the propeptide, while the asterisk marks the first amino acid residue of the mature CmCatD based on sequence alignment.

quantitative RT-PCR analysis. The 1st instar was excluded due to its minute size, which rendered midgut dissection unfeasible. Under unchallenged conditions, *CmCatD* transcript abundance in 4th instar was significantly lower than that of the earlier stages (Fig. 2), indicating that *CmCatD* expression was developmentally controlled. This profile, however, was altered by dietary scN; following a down-regulation under scN in early development, the mRNA increased in the 3rd instar to a level similar to the control 2nd instar, and maintained this through the 4th instar (Fig. 2). Contrast between the 4th instars of the two groups is in agreement with the microarray results, where higher expression of *CmCatD* in scN-fed than control 4th instar insects was detected (Chi et al., 2009). This pattern also correlates well with the differential proteolytic activity observed (Zhu-Salzman et al., 2003).

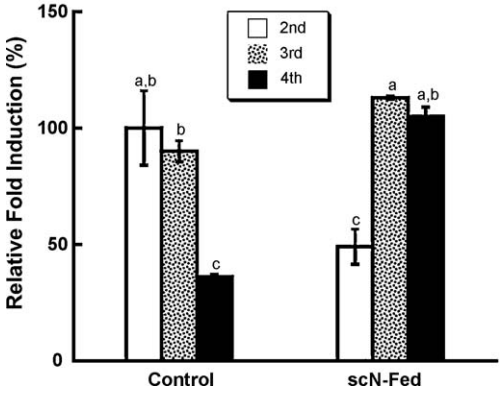

Fig. 2. Dietary scN altered developmentally regulated *CmCatD* expression. Quantitative RT-PCR was performed to profile *CmCatD* expression during larval development in the presence and absence of scN challenge. Total RNA was extracted from midguts of cowpea bruchids fed on control or scN-containing diet, respectively, at larval developmental stages indicated. The control 2nd instar mRNA level was arbitrarily set at 100% for calculation of relative transcript abundance. Each bar represents the mean fold induction ±S.D. ($n = 3$). Means are not significantly different when followed by the same letter as determined by Fisher's LSD test ($P = 0.01$).

3.2. Soluble expression of CmCatD

In order to illustrate the functionality of CmCatD in insect counter-defense against dietary scN, we bacterially expressed the recombinant protein with the signal peptide removed. However, the hexa-His tagged proprotein (proCmCatD) was insoluble, suggesting that this heterologously expressed protein was improperly folded. Lower induction temperatures did not improve the solubility (data not shown). We then applied guanidine and urea solutions to the recombinant protein, followed by Ni^{2+}-affinity purification under denaturing conditions (Holzinger et al., 1996). After removal of denaturing reagents, a small portion of the expressed recombinant protein, approximately 1 mg from 500 ml bacterial culture, was recovered as soluble protein (Fig. 3). Nevertheless, recovery of a portion of the protein in soluble form made possible further functional characterization of CmCatD.

3.3. pH-dependent autoprocessing and proteolytic activities

Cathepsin D-like aspartic proteases are synthesized as proteolytically inactive proproteins that are inhibited by their own N-terminal propeptides. Acidification triggers autocatalytic removal of the propeptide, activating the enzyme (Dunn, 2002). Autoprocessing of proCmCatD was evaluated between pH 3.0 and 6.0. At the lower pH range, incubation at 37 °C for 1 h converted the 43 kDa recombinant proprotein to a shorter, mature CmCatD form (Fig. 4A). As expected, the aspartic protease inhibitor pepstatin A prevented this conversion (Fig. 4B), as this inhibitor is able to bind to the active site of the cathepsin D proprotein blocking the self-catalyzed intramolecular proteolysis (Beyer and Dunn, 1996). Intensity of the mature CmCatD band decreased as pH became more acidic, suggesting self-degradation had occurred likely due to structural instability at low pH.

To determine the optimal pH for enzymatic activity of mature CmCatD, we used hemoglobin as the substrate. Maximal hydrolysis of hemoglobin was detected at pH 4.0 (Fig. 5A), consistent with the previously reported pH at which aspartic protease activity was detected from cowpea bruchid midgut (Silva and Xavier-Filho,

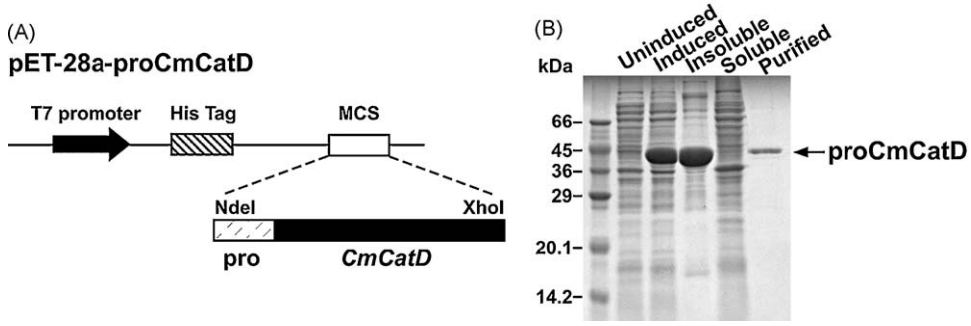

Fig. 3. Bacterial expression of CmCatD proprotein. (A) Diagram of pET28a-*proCmCatD* construct. His tag: hexa-His for purification of the recombinant protein through Ni^{2+}-chelating affinity chromatography. pro: propeptide region. CmCatD: mature enzyme region. (B) SDS-PAGE. Production of recombinant proCmCatD was induced with 1 mM IPTG following transfer of the construct into *E. coli* BL21 (DE3) host cells. The expressed protein was insoluble and thus had to be purified under denaturing conditions, then renatured. Proteins extracted from whole cells as well as the purified proCmCatD were analyzed on 12.5% SDS-PAGE.

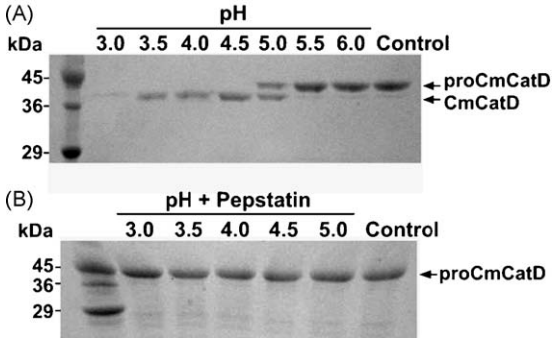

Fig. 4. *In vitro* autoprocessing of the recombinant proCmCatD. (A) Effect of pH on autoprocessing. proCmCatD was incubated at 37 °C for 1 h under pH conditions indicated. The reactions were then resolved on 12.5% SDS-PAGE. proCmCatD without incubation was used as a control. (B) Pepstatin A abolishes autoprocessing activity of proCmCatD. The proCmCatD was incubated with pepstatin A at room temperature for 20 min prior to incubation at 37 °C under the indicated pH for 1 h. The samples were then subjected to 12.5% SDS-PAGE.

1991). Mature CmCatD remained active in a fairly wide pH range. Further, the hemoglobin-hydrolyzing activity of CmCatD was specifically inhibited by pepstatin A, while unaffected by inhibitors specific for serine and cysteine proteases (Fig. 5B).

3.4. Coordinated mutual trans-activation with major digestive CmCPs

Autoprocessing of CmCatD became less efficient and eventually diminished at pH values 5.0 and above (Fig. 4A), near the physiological pH which is optimal for cysteine proteases. At pH 5.5, while autoprocessing was inactive, mature CmCatD exhibited approximately 50% of its maximal enzymatic activity (Fig. 5A). It is likely that activation of CmCatD *in vivo* may rely on the most abundant midgut CmCP proteases. To investigate the potential transactivation of proCmCatD by a major digestive CmCP, we incubated mature CmCPB1 with proCmCatD at pH 5.0, 5.5 and 6.0 (Fig. 6A). CmCPB1, a highly active cysteine protease isoform capable of autoprocessing, was selectively induced by dietary scN (Zhu-Salzman et al., 2003). Appearance of a protein band with a molecular weight of mature CmCatD paralleled the observed loss of proCmCatD, suggesting that transactivation by CmCP is possible in cowpea bruchid midgut. The processed protein appeared stable as no apparent degradation was observed. Therefore, near the midgut physiological pH where CmCatD autoprocessing did not occur, proCmCatD could be trans-processed by CmCPB1, and under such a pH (5.5), the matured CmCatD could exhibit substantial activity (Fig. 5A).

Among major digestive CmCP isoforms, CmCPA16 is incapable of autoprocessing (Ahn et al., 2004). Previously, we showed that bruchid gut aspartic protease activity contributes to activation of CmCPA16. To determine if CmCatD could be responsible for this trans-activation, autoprocessed CmCatD was incubated with proCmCPA16. The proprotein band was processed into two fragments, whose molecular sizes corresponded to the mature CmCPA16 and its propeptide (Fig. 6B). Coincidentally, mature CmCatD and proCmCP16 co-migrated, as did mature CmCPA16 and a bacterial protein (co-purified with proCmCPA16). Anti-CmCP antibody was used to distinguish CmCPA16 from others (Fig. 6B). Results indicated that CmCatD can trans-process CmCPA16, thus activating this enzyme that cannot self-activate.

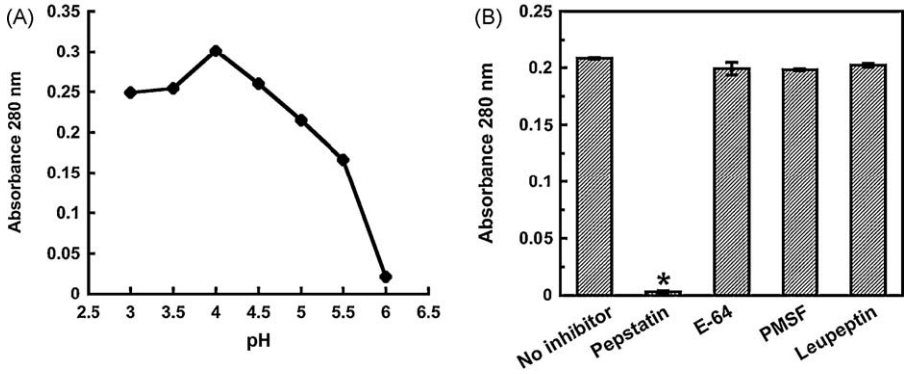

Fig. 5. Proteolytic activity of CmCatD and inhibition. (A) Proteolytic activity of CmCatD is pH-dependent. Hemoglobin hydrolysis was used to measure the proteolytic activity of autoprocessed CmCatD under pH conditions indicated. Experiments were done in triplicate. Average absorbance at 280 nm resulting from hemoglobin hydrolysis by CmCatD. (B) Enzymatic activity of CmCatD is specifically inhibited by the aspartic protease inhibitor. The autoprocessed CmCatD was pre-incubated with water or inhibitors specific for aspartic (pepstatin A), cysteine (E-64), serine (PMSF), or serine/cysteine proteases (leupeptin), respectively, and hemoglobin-hydrolyzing activity was then measured in triplicate. The asterisk indicates that the mean is significantly different from others (Fisher's LSD test, $P = 0.01$). Error bars indicate standard error.

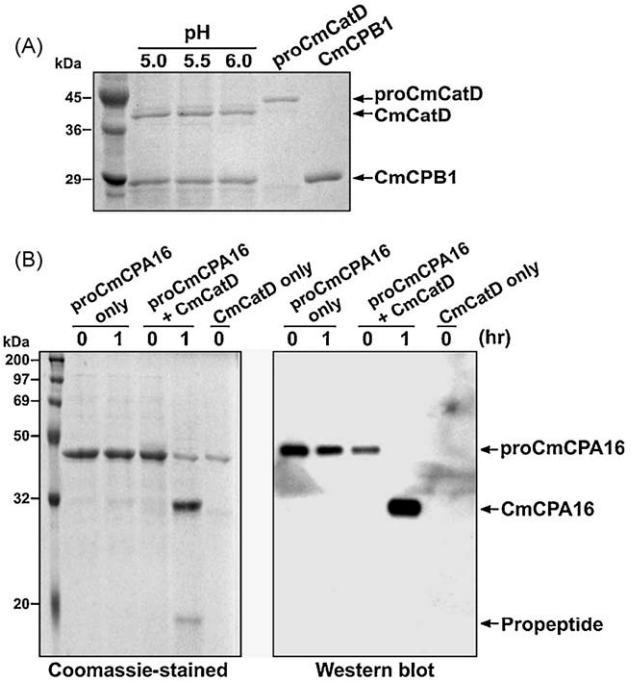

Fig. 6. Mutual trans-processing between bruchid digestive protease families. (A) Trans-activation of proCmCatD by an scN-induced, cathepsin L-like CmCPB1. The proCmCatD was incubated with autoprocessed CmCPB1 at 37 °C for 1 h at indicated pH, and then subjected to separation on 12.5% SDS-PAGE. (B) CmCatD trans-processes proCmCPA16, a CmCP isoform that lacks autoprocessing. Autoprocessed CmCatD was incubated with proCmCPA16 in 100 mM sodium acetate, pH 5.0 for 0 or 1 h at 37 °C, respectively. The samples were then analyzed on 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue R-250, or transferred to PVDF membrane for immunoblot analysis using anti-recombinant CmCP antibody.

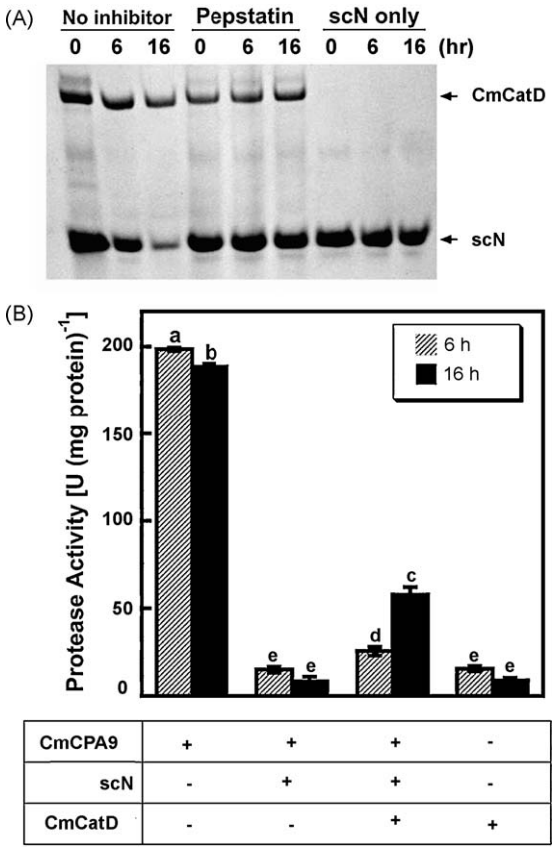

Fig. 7. CmCatD degraded scN and facilitated CmCP activity. (A) Hydrolysis of scN CmCatD. The autoprocessed CmCatD, pre-incubated with water or pepstatin A, was mixed with scN for a further incubation of 0, 6 and 16 h respectively at 37 °C. The samples were then analyzed on 15% tricine SDS-PAGE. (B) The presence of CmCatD freed a portion of CmCPA9 otherwise inhibited by scN. The processed CmCatD and CmCPA9 as well as scN were incubated at a 1:1:1 molar ratio for 0, 6 or 16 h at 37 °C. The enzymatic activity of CmCPA9 on a synthetic peptide Z-Phe-Arg-pNA, a cathepsin L-specific substrate, was measured in triplicate, and compared with that in the absence of CmCatD. CmCPA9 and CmCatD alone served as the positive and negative controls. Means are not significantly different when followed by the same letter as determined by Fisher's LSD test ($P = 0.01$). Error bars indicate standard errors.

3.5. CmCatD hydrolyzes scN

Plant defense proteins often are resistant to digestion in the insect gut, allowing them to reach their target sites. In fact, scN remained intact for an extended period of time, when incubated with a midgut extract of scN-unadapted 4th instar bruchid larvae. Yet, gut extract obtained from the 4th instar larvae reared on scN-containing diet could partially degrade scN. Among various scN-induced changes was an increase in aspartic protease activity (Zhu-Salzman et al., 2003). To test whether CmCatD could contribute to the scN-degrading activity, we incubated it with scN at the optimal pH for CmCatD for different time periods. scN degradation became apparent after 6 h incubation (Fig. 7A). Sustained expression of CmCatD through the 4th instar (Fig. 2), coupled with increased expression of the major digestive CmCPs, potentially facilitated counter-defense against dietary scN in cowpea bruchids.

To determine the impact of CmCatD on scN inhibition of CmCP proteolytic activity, we pre-incubated CmCPA9, CmCatD and scN at a molar ratio of 1:1:1 prior to addition of Z-Phe-Arg-pNA, a synthetic chromogenic substrate specific for cysteine proteases. The reaction exhibited partial proteolysis, in contrast to total inhibition of CmCPA9 activity when CmCatD was absent (Fig. 7B). Thus, scN degradation by CmCatD presumably decreased the concentration of the inhibitor that could actively block CmCPA9 activity. As a result, some CmCPA9 molecules became available for substrate hydrolysis.

4. Discussion

The aspartic protease cathepsin D functions in the intracellular and extracellular degradation of proteins. In insects, it has been shown to be important in metamorphosis and food degradation. Here, we demonstrate its potential functionality in helping insects cope with dietary challenge, possibly via its cooperation with major digestive cysteine proteases. When insects were reared on regular diet, expression of *CmCatD* decreased as bruchid larvae developed (Fig. 2). Such a profile, although in marked contrast to the major digestive *CmCP* proteases that expressed steadily throughout the larval development (Moon et al., 2004), has been reported in other insect cathepsin D genes (Gui et al., 2006). Presumably in these cases, high abundance of *CatD* transcripts in the midgut of late stage larvae is unnecessary under normal growth conditions. However, this developmentally regulated gene expression is subject to alteration by dietary scN (Fig. 2). Increased extracellular, but not intracellular, procathepsin D abundance has been shown in mammalian cell lines when treated with inflammatory cytokines produced from the vertebrate immune system (Erdmann et al., 2008). Differential regulation of *CmCatD* can be viewed as a counter-defense response to a dietary challenge.

A remarkable feature of the insect midgut lies in its ability to reconfigure the digestive enzyme complement, leading to decreased scN impact. The predominant digestive CmCPs are strongly induced to compensate for inhibited activity. Among these, CmCPB1 is the preferentially induced, highly active isoform, however its activity is susceptible to scN inhibition (Ahn et al.,

2004). Higher expression of the normally minor CmCatD could be important when the major digestive CmCPs are inhibited because (i) CmCatD contributes to more proteolysis of dietary proteins due to its insensitivity to scN and (ii) its ability to hydrolyze scN not only decreases the concentration of scN, but converts it into building blocks for new protein synthesis, benefiting the insects themselves.

It has been shown that human cathepsin D can cleave and thus inactivate cysteine protease inhibitors, both intracellularly and extracellularly (Lenarcic et al., 1991). Cathepsin D from Colorado potato beetle initiated hydrolysis of a dietary cysteine protease inhibitor, which was completed by a subsequent action of cysteine and serine proteases (Brunelle et al., 1999). scN-induced and scN-hydrolyzing enzymatic activity has been detected in cowpea bruchid alimentary tract (Zhu-Salzman et al., 2003). Cathepsin D most likely contributes to this activity, thus playing a role in regulating midgut digestive capacity.

Although it was eventually degraded by recombinant CmCatD, scN was somewhat resistant to hydrolysis, judging by the incubation time required for degradation (Fig. 7A). This refractory nature is expected from an effective plant defensive protein, because it must survive the often hostile chemical milieu of the insect alimentary tract in order to exert its function (Chen et al., 2007; Zhu-Salzman et al., 2008). Not all defensive proteins can survive intact. For instance, wheat alpha amylase inhibitor and Kunitz inhibitor had little or no effect on cowpea bruchids if they were administered alone, but when combined with scN, a synergistic effect was achieved. Resistance of scN to proteolysis prevented degradation of the alpha amylase inhibitor or Kunitz inhibitor by the major digestive cysteine proteases, allowing them to reach the target sites (Amirhusin et al., 2004, 2007). Action of CmCatD on scN, although rather slow, is likely beneficial to insects in the presence of this dietary inhibitor by lowering the effective inhibitor concentration, which in turn partially frees cysteine proteases for food protein hydrolysis.

Cooperation between CmCatD and CmCPs is also reflected by their mutual trans-activation during propeptide processing without compromising structural integrity of either class (Fig. 6). Activation of proCmCatD by CmCPB1 into mature CmCatD should be particularly valuable, considering its suboptimal autoprocessing at physiological pH. Cysteine protease-assisted conversion has been shown for human procathepsin D, a process independent of autoactivation (Laurent-Matha et al., 2006). Likewise, it could be equally important for CmCatD to be able to trans-process those major digestive CmCP isoforms, such as CmCPA16, that lack self-maturing capability (Ahn et al., 2004). Ineffective autoprocessing may not affect their activation under a normal feeding environment, as activated CmCPs could process the proproteins. However, under dietary scN challenge, where the normally available CmCPs are inhibited, an inability to self-process could become an obstacle to digestive function. Since CmCatD is scN-insensitive, its ability to process CmCP proproteins presumably helps increase the quantity of active digestive enzymes. Results in this study are in excellent agreement with our previous observation that bruchid gut aspartic protease activity also could contribute to activation of CmCPA16 (Ahn et al., 2004).

Coordination and complementation between the two classes of digestive proteases presumably increase digestive capacity and enable insects to effectively cope with dietary threats. Although uninvestigated in the cowpea bruchid, compartmentalization in the insect midgut is postulated to accommodate the presence of more than one mechanistic class of digestive enzymes (Terra and Ferreira, 1994). Many insect species including coleopterans have a range of midgut pH, from acidic to alkaline in separate regions of the gut (Oppert et al., 1993; Terra and Ferreira, 1994; Edmonds et al., 1996). In one scenario, when the main digestive machinery is impaired by scN, the aspartic proteases could continue the protein digestion process in a localized, more acidic area, assisting cowpea bruchids in overcoming temporary nutrient deficiencies. On the other hand, co-existence of two classes of enzymes in the physiological environment, with the pH value more suitable for cysteine proteases, could be crucial for insects under dietary challenge because cathepsin D is able to activate CmCPs. Also, it should be mentioned that the pH optimum for CmCatD was determined based on the *in vitro* setting. Molecular interactions with certain physiological activators, however, have been shown to enable cathepsin D to effectively cleave proteins at neutral pH (Lkhider et al., 2004). It remains speculative whether such activators exist in the bruchid midgut, but it is possible that *in vivo* CmCatD hydrolysis is more efficient than we observed.

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References

Ahn, J.E., Salzman, R.A., Braunagel, S.C., Koiwa, H., Zhu-Salzman, K., 2004. Functional roles of specific bruchid protease isoforms in adaptation to a soybean protease inhibitor. Insect Molecular Biology 13, 649–657.

Amirhusin, B., Shade, R.E., Koiwa, H., Hasegawa, P.M., Bressan, R.A., Murdock, L.L., Zhu-Salzman, K., 2004. Soyacystatin N inhibits proteolysis of wheat alpha-amylase inhibitor and potentiates toxicity against cowpea weevil. Journal of Economic Entomology 97, 2095–2100.

Amirhusin, B., Shade, R.E., Koiwa, H., Hasegawa, P.M., Bressan, R.A., Murdock, L.L., Zhu-Salzman, K., 2007. Protease inhibitors from several classes work synergistically against *Callosobruchus maculatus*. Journal of Insect Physiology 53, 734–740.

Barrett, A.J., 1970. Cathepsin D—purification of isoenzymes from human and chicken liver. Biochemical Journal 117, 601–607.

Beyer, B.M., Dunn, B.M., 1996. Self-activation of recombinant human lysosomal procathepsin D at a newly engineered cleavage junction, "short" pseudocathepsin D. Journal of Biological Chemistry 271, 15590–15596.

BlancoLabra, A., MartinezGallardo, N.A., SandovalCardoso, L., DelanoFrier, J., 1996. Purification and characterization of a digestive cathepsin D proteinase isolated from *Tribolium castaneum* larvae (Herbst). Insect Biochemistry and Molecular Biology 26, 95–100.

Boldbaatar, D., Sikasunge, C.S., Battsetseg, B., Xuan, X., Fujisaki, K., 2006. Molecular cloning and functional characterization of an aspartic protease from the hard tick *Haemaphysalis longicornis*. Insect Biochemistry and Molecular Biology 36, 25–36.

Brunelle, F., Nguyen-Quoc, B., Cloutier, C., Michaud, D., 1999. Protein hydrolysis by Colorado potato beetle, *Leptinotarsa decemlineata*, digestive proteases: the catalytic role of cathepsin D. Archives of Insect Biochemistry and Physiology 42, 88–98.

Chen, H., Gonzales-Vigil, E., Wilkerson, C.G., Howe, G.A., 2007. Stability of plant defense proteins in the gut of insect herbivores. Plant Physiology 143, 1954–1967.

Chi, Y.H., Salzman, R.A., Balfe, S., Ahn, J.E., Sun, W., Moon, J., Yun, D.J., Lee, S.Y., Higgins, T.J., Pittendrigh, B., Murdock, L.L., Zhu-Salzman, K., 2009. Cowpea bruchid midgut transcriptome response to a soybean cystatin—costs and benefits of counter-defence. Insect Molecular Biology 18, 97–110.

Cho, W.L., Raikhel, A.S., 1992. Cloning of cDNA for mosquito lysosomal aspartic protease—sequence-analysis of an insect lysosomal-enzyme similar to cathepsin D and E. Journal of Biological Chemistry 267, 21823–21829.

Cloutier, C., Jean, C., Fournier, M., Yelle, S., Michaud, D., 2000. Adult Colorado potato beetles, *Leptinotarsa decemlineata* compensate for nutritional stress on oryzacystatin I-transgenic potato plants by hypertrophic behavior and over-production of insensitive proteases. Archives of Insect Biochemistry and Physiology 44, 69–81.

De Leo, F., Bonade-Bottino, M.A., Ceci, L.R., Gallerani, R., Jouanin, L., 1998. Opposite effects on *Spodoptera littoralis* larvae of high expression level of a trypsin proteinase inhibitor in transgenic plants. Plant Physiology 118, 997–1004.

Dunn, B.M., 2002. Structure and mechanism of the pepsin-like family of aspartic peptidases. Chemical Reviews 102, 4431–4458.

Edmonds, H.S., Gatehouse, L.N., Hilder, V.A., Gatehouse, J.A., 1996. The inhibitory effects of the cysteine protease inhibitor, oryzacystatin, on digestive proteases and on larval survival and development of the southern corn rootworm (*Diabrotica undecimpunctata* howardi). Entomologia Experimentalis Et Applicata 78, 83–94.

Englard, S., Seifter, S., 1990. Precipitation techniques. Methods in Enzymology 182, 285–300.

Erdmann, S., Ricken, A., Hummitzsch, K., Merkwitz, C., Schliebe, N., Gaunitz, F., Strotmann, R., Spanel-Borowski, K., 2008. Inflammatory cytokines increase extracellular procathepsin D in permanent and primary endothelial cell cultures. European Journal of Cell Biology 87, 311–323.

Faust, P.L., Kornfeld, S., Chirgwin, J.M., 1985. Cloning and sequence-analysis of cDNA for human cathepsin D. Proceedings of the National Academy of Sciences of the United States of America 82, 4910–4914.

Gruden, K., Kuipers, A.G.J., Guncar, G., Slapar, N., Strukelj, B., Jongsma, M.A., 2004. Molecular basis of Colorado potato beetle adaptation to potato plant defence at the level of digestive cysteine proteinases. Insect Biochemistry and Molecular Biology 34, 365–375.

Gui, Z.Z., Lee, K.S., Kim, B.Y., Choi, Y.S., Wei, Y.D., Choo, Y.M., Kang, P.D., Yoon, H.J., Kim, I., Je, Y.H., Seo, S.J., Lee, S.M., Guo, X.J., Sohn, H.D., Jin, B.R., 2006. Functional role of aspartic proteinase cathepsin D in insect metamorphosis. BMC Developmental Biology 6, 49, doi:10.1186/1471-213-6-49.

Holzinger, A., Phillips, K.S., Weaver, T.E., 1996. Single-step purification solubilization of recombinant proteins: application to surfactant protein B. Biotechniques 20, 804–808.

Jongsma, M.A., Bakker, P.L., Peters, J., Bosch, D., Stiekema, W.J., 1995. Adaptation of *Spodoptera exigua* larvae to plant proteinase-inhibitors by induction of gut proteinase activity insensitive to inhibition. Proceedings of the National Academy of Sciences of the United States of America 92, 8041–8045.

Jongsma, M.A., Bolter, C., 1997. The adaptation of insects to plant protease inhibitors. Journal of Insect Physiology 43, 885–895.

Kitch, L.W., Murdock, L.L., 1986. Partial characterization of a major gut thiol proteinase from larvae of *Callosobruchus maculatus* F. Archives of Insect Biochemistry and Physiology 3, 561–575.

Laurent-Matha, V., Derocq, D., Prebois, C., Katunuma, N., Liaudet-Coopman, E., 2006. Processing of human cathepsin D is independent of its catalytic function and auto-activation: involvement of cathepsins L and B. Journal of Biochemistry 139, 363–371.

Lenarcic, B., Krasovec, M., Ritonja, A., Olafsson, I., Turk, V., 1991. Inactivation of human cystatin C and kininogen by human cathepsin D. FEBS Letters 280, 211–215.

Liu, Y.L., Salzman, R.A., Pankiw, T., Zhu-Salzman, K., 2004. Transcriptional regulation in southern corn rootworm larvae challenged by soyacystatin N. Insect Biochemistry and Molecular Biology 34, 1069–1077.

Lkhider, M., Castino, R., Bouguyon, E., Isidoro, C., Ollivier-Bousquet, M., 2004. Cathepsin D released by lactating rat mammary epithelial cells is involved in prolactin cleavage under physiological conditions. Journal of Cell Science 117, 5155–5164.

Mazumdar-Leighton, S., Broadway, R.M., 2001. Transcriptional induction of diverse midgut trypsins in larval *Agrotis ipsilon* and *Helicoverpa zea* feeding on the soybean trypsin inhibitor. Insect Biochemistry and Molecular Biology 31, 645–657.

Moon, J., Salzman, R.A., Ahn, J.E., Koiwa, H., Zhu-Salzman, K., 2004. Transcriptional regulation in cowpea bruchid guts during adaptation to a plant defence protease inhibitor. Insect Molecular Biology 13, 283–291.

Oppert, B., Morgan, T.D., Culbertson, C., Kramer, K.J., 1993. Dietary mixtures of cysteine and serine proteinase-inhibitors exhibit synergistic toxicity toward the red flour beetle, *Tribolium castaneum*. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology 105, 379–385.

Rabossi, A., Stoka, V., Puizdar, V., Turk, V., Quesada-Allue, L.A., 2004. Novel aspartyl proteinase associated to fat body histolysis during *Ceratitis capitata* early metamorphosis. Archives of Insect Biochemistry and Physiology 57, 51–67.

Schagger, H., Vonjagow, G., 1987. Tricine sodium dodecyl-sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1-kDa to 100-kDa. Analytical Biochemistry 166, 368–379.

Silva, C.P., Xavier-Filho, J., 1991. Comparison between the levels of aspartic and cysteine proteinases of the larval midguts of *Callosobruchus maculatus* (F) and *Zabrotes subfasciatus* (Boh) (Coleoptera, Bruchidae). Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 99, 529–533.

Tcherepanova, I., Bhattacharyya, L., Rubin, C.S., Freedman, J.H., 2000. Aspartic proteases from the nematode *Caenorhabditis elegans*—structural organization and developmental and cell-specific expression of *asp-1*. Journal of Biological Chemistry 275, 26359–26369.

Terra, W.R., Ferreira, C., 1994. Insect digestive enzymes—properties, compartmentalization and function. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 109, 1–62.

Yang, L., Fang, Z., Dicke, M., van Loon, J.J., Jongsma, M.A., 2009. The diamondback moth, *Plutella xylostella*, specifically inactivates Mustard Trypsin Inhibitor 2 (MTI2) to overcome host plant defence. Insect Biochemistry and Molecular Biology 39, 55–61.

Zhu-Salzman, K., Koiwa, H., Salzman, R.A., Shade, R.E., Ahn, J.E., 2003. Cowpea bruchid *Callosobruchus maculatus* uses a three-component strategy to overcome a plant defensive cysteine protease inhibitor. Insect Molecular Biology 12, 135–145.

Zhu-Salzman, K., Luthe, D.S., Felton, G.W., 2008. Arthropod-inducible proteins, broad spectrum defenses against multiple herbivores. Plant Physiology 146, 852–858.

Zhu-Salzman, K., Salzman, R.A., 2001. Functional mechanics of the plant defensive *Griffonia simplicifolia* lectin II: resistance to proteolysis is independent of glycoconjugate binding in the insect gut. Journal of Economic Entomology 94, 1280–1284.