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Inhibition of cysteine and aspartyl proteinases in the alfalfa weevil midgut with biochemical and plant-derived proteinase inhibitors

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Abstract

Proteolytic activities in alfalfa weevil (*Hypera postica*) larval midguts have been characterized. Effects of pH, thiol activators, low-molecular weight inhibitors, and proteinase inhibitors (PIs) on general substrate hydrolysis by midgut extracts were determined. Hemoglobinolytic activity was highest in the acidic to mildly acidic pH range, but was maximal at pH 3.5. Addition of thiol-activators dithiothreitol (DTT), 2-mercaptoethanol (2-ME), or L-cysteine had little effect on hemoglobin hydrolysis at pH 3.5, but enhanced azocaseinolytic activity two to three-fold at pH 5.0. The broad cysteine PI E-64 reduced azocaseinolytic activity by 64% or 42% at pH 5 in the presence or absence of 5 mM L-cysteine, respectively. Inhibition by diazomethyl ketones, Z-Phe–Phe–CHN_2 and Z-Phe–Ala–CHN_2, suggest that cathepsins L and B are present and comprise approximately 70% and 30% of the cysteine proteolytic activity, respectively. An aspartyl proteinase component was identified using pepstatin A, which inhibited 32% (pH 3.5, hemoglobin) and 50% (pH 5, azocasein) of total proteolytic activity. This activity was completely inhibited by an aspartyl proteinase inhibitor from potato (API), and is consistent with the action of a cathepsin D-like enzyme. Hence, genes encoding PIs with specificity toward cathepsins L, B and D could potentially be effective for control of alfalfa weevil using transgenic plants.

Keywords: Coleoptera; Midgut proteinases; *Hypera postica*; Cathepsin L; Cathepsin D

1. Introduction

The use of genes encoding proteinase inhibitors (PIs) to transform crop plants for resistance to insect pests has been well documented (for reviews, see Jouanin et al., 1998; Schuler et al., 1998). PIs occur naturally in a number of plant species and are believed to have a role in the natural defense of plants against insect pests (Green and Ryan, 1972). The ability of PIs to interfere with insect growth and development has been attributed to their capacity to bind to, and thereby inhibit the action of insect digestive proteinases (reviewed by Jongsma and Bolter, 1997). Since there exists significant variation among the types and properties of endo- and exo-proteinases utilized by insects for dietary purposes (reviewed by Terra and Ferreira, 1994), and because numerous PIs of widely-ranging specificities (Keilova and Tomasek, 1976a; Abe et al., 1994; Brzin et al., 1998; Christeller et al., 1998; Pernas et al., 1998) towards these proteinases are available, it is necessary to characterize the gut proteolytic activities of each individual species in order to devise a rational insect control strategy utilizing PIs.

There is a heavy reliance among insects on endoproteinases for the purpose of assimilating dietary protein. Endoproteinases belong to one of four groups based on the amino acid residue or metal ion involved in peptide bond catalysis (Barrett, 1986): (i) serine, (ii) cysteine (or thiol), (iii) aspartyl (or carboxyl), and (iv) metalloproteinases. Insects having a midgut pH in the neutral or alkaline range generally rely on serine proteinases that are trypsin-, chymotrypsin-, or elastase-like, based on their similarity to mammalian enzymes. In contrast, insects having acidic or mildly acidic midguts generally utilize cysteine proteinases, such as cathepsins B, H and

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L, and/or aspartyl proteinases such as cathepsin D and pepsin (Terra and Ferreira, 1994). While there exists a pattern of predominance involving serine proteinases in Diptera and Lepidoptera (Applebaum, 1985; Christeller et al., 1992; Terra and Ferreira, 1994), and cysteine proteinases in Coleoptera (Murdock et al., 1987), exceptions are common. Of particular relevance here, Coleoptera have been demonstrated to also utilize serine and aspartyl proteinases (Christeller et al., 1989; Thie and Houseman, 1990; Bonade-Bottino et al., 1999). Such complexity that often involves two or more proteinase classes necessitates the use of multiple PIs for insect control, as uninhibited proteinases can degrade the ingested PI and compensate for the activity lost as a result of PI action on sensitive proteinases (Broadway, 1996a; Jongsma and Bolter, 1997; Girard et al., 1998a,b).

This study characterizes the major midgut proteinases of larval alfalfa weevil, *Hypera postica* Gyllenhal (Coleoptera: Curculionidae), a major pest of alfalfa for which traditional breeding techniques for resistance have failed. The effect of pH, low-molecular weight inhibitors, and plant-derived PIs on proteinase activity in midgut extracts is presented.

2. Materials and methods

2.1. Materials

Azocasein, hemoglobin, L-*trans*-epoxysuccinyl–leucylamido(4-guanidino)butane (E-64), leupeptin, pepstatin A, phenylmethylsulfonyl fluoride (PMSF), and soybean trypsin–chymotrypsin inhibitor (Bowman-Birk inhibitor I, BBI) were from Sigma Chemical Co. (St. Louis, MO). Peptidyl–diazomethane inhibitors, Z-Phe–Phe–CHN_2 and Z-Phe–Ala–CHN_2, were obtained from Bachem California (Torrance, CA). Oryzacystatin I (OCI) (Abe et al., 1987) was expressed as s glutathione S-transferase (GST) fusion protein in *Escherichia coli* strain BL21 (Stratagene) as previously described (Michaud et al., 1994). Aspartyl proteinase inhibitor (API) was purified from potato as previously described (Kreft et al., 1997) and consisted of several isoforms (Ritonja et al., 1990; Barlic-Maganja et al., 1992; Strukelj et al. 1992, 1995; Kreft et al., 1997) of potato cathepsin D inhibitor (PDI) (Keilova and Tomasek, 1976b).

2.2. Insects

Alfalfa weevil larvae used in this study were second-generation laboratory-reared, derived from eggs of non-diapausing field-collected adults, as described by Ratcliffe and Elgin (1987). To assure an ample supply of eggs, successive generations of newly-emerged adults were fed bouquets of greenhouse-grown alfalfa foliage for 2 wk, and then placed in cold storage (4°C) on 2% (w/v) sugar water for more than 8 wk. After removal from storage, adults were fed alfalfa foliage for 3 wk, after which time they began oviposition (Elden, 1995a). Larvae were reared on flats of greenhouse-grown alfalfa. All insects were reared in a walk-in environmental growth chamber maintained under a photoperiod of 8:16 (L:D) h at 24±1°C and 50–90% r.h.

Alfalfa weevil midguts were excised from late third and early fourth instar larvae under magnification. After splitting the body cavity lengthwise, the midgut was removed excluding the fore- and hindgut. Only full guts containing visible contents were used. Midguts were immediately placed in a micro-eppendorf tube embedded in dry ice. Samples consisting of 50 midguts were frozen at −80°C for later extract preparation.

2.3. Midgut extract preparation

This procedure was modified from Michaud et al. (1995). Frozen midguts were thawed on ice and homogenized following the addition of ice-cold citrate–phosphate buffer (pH 6.0) containing 0.1% Triton X-100. Homogenates were microfuged at 16,000***g*** for 5 min at 4°C. Supernatants were transferred to fresh tubes and pellets re-extracted as above. Pooled supernatants were microfuged one final time for 30 min at 4°C. Cleared supernatants were concentrated, when necessary, using microcon-3 microconcentrators (Amicon, Inc., Beverly, MA) and applied to Bio-Gel P6 columns (Bio-Rad, Hercules, CA) at 100 μl per column. Flow-through was collected, aliquoted, and stored at −20°C following protein determination using the Bio-Rad Protein Assay with bovine serum albumin (BSA) as a standard. Only residual proteinase activity (<1% of total) was detected in the pellet following this extraction method.

2.4. Proteinase and PI assays

Azocaseinase determinations were adapted from Sarath et al. (1989) and Barrett and Kirschke (1981) and Barrett and Kirschke (1981). Enzyme extracts (containing from 5–20 μg protein) were combined with 5 μl of 2.4% Triton X-100, 34 μg of BSA, and 100 mM citrate–phosphate buffer at the appropriate pH to yield 40 μl. Eighty-microliters of 2% (w/v) azocasein (prepared in citrate–phosphate buffer of desired pH) was added, and the final reaction mix was incubated for 3 h at 37°C. The final concentration of reactants was 0.1% Triton X-100, 0.028% (w/v) BSA, and 1.33% (w/v) azocasein in the 120 μl reaction. Reactions were terminated by adding 300 μl of 10% (w/v) trichloroacetic acid. Samples were incubated on ice for 10 min followed by sedimentation of undigested substrate by centrifugation at 16,000***g*** for 5 min at room temperature (RT). Super-

natants (~380 μl) containing TCA-soluble peptide fragments were transferred to methacrylate cuvettes (Fisher Scientific) and their absorbance (*A*) measured at 335 nm using a Beckman DU-70 spectrophotometer. The A_{335} of blanks incubated for 3 h at 37°C, which consisted of complete reaction mixtures minus extract, were used for background subtraction to account for spontaneous substrate degradation.

Activators and PIs were included, when appropriate, in the 40 μl reaction mix prior to substrate addition. Concentrations of activators and inhibitors refer to their concentration in the final 120 μl reaction. For inhibition assays, the 40 μl reaction mix was pre-incubated approximately 5 min at 37°C to allow for the proteinase-inhibitor interaction prior to substrate addition.

Due to the insolubility of azocasein below pH 4.5, hemoglobin was used as the substrate for determination of pH optimum and for thiol-activation and inhibitor assays carried out at pH 3.5. The assay for hemoglobin proteolysis was based on the method of Houseman and Downe (1983). It was conducted in the same manner as the azocasein assay with the following modifications. The volume of reactants prior to the addition of 40 μl of 2% (w/v) hemoglobin (prepared in 0.15 M NaCl) was 80 μl. Thus, the final concentration of reactants was 0.1% Triton X-100, 0.028% (w/v) BSA, and 0.67% (w/v) hemoglobin in the 120 μl reaction mix. Reactions were terminated by the addition of 300 μl trichloroacetic acid and the absorbance measured at 280 nm. We define one unit of enzyme activity as being the amount of enzyme that will produce an absorbance change of 1.0 h^{-1} in a 1 cm cuvette, under the conditions of the assay. Preliminary experiments were conducted to ensure that all determinations were made within the linear range (by plotting extract quantity vs absorbance) of the assay using both substrates.

3. Results

3.1. Proteolytic activity in alfalfa weevil midguts

Proteolytic activity in extracts from weevil larvae was measured over pH values ranging from 2.5 to 10.0 (Fig. 1) with hemoglobin as substrate. The overall pattern of activity observed indicates the presence of proteolytic enzymes active in the acidic and mildly-acidic range. This is consistent with aspartyl and cysteine proteinases that generally have pH optimums in the range of 2–5 and 4–7, respectively. Serine and metalloproteinases have optimums at pH values of 7–9 (Barrett, 1977a; Storey and Wagner, 1986) and, therefore, do not appear to be major constituents in the extract. Maximal activity using this assay was observed at pH 3.5 with approximately 50% of the activity remaining at pH 5.0. The absence of any measurable hemoglobinolytic activity at pH ≥7 is consistent with what we have also observed utilizing azocasein as substrate (data not shown).

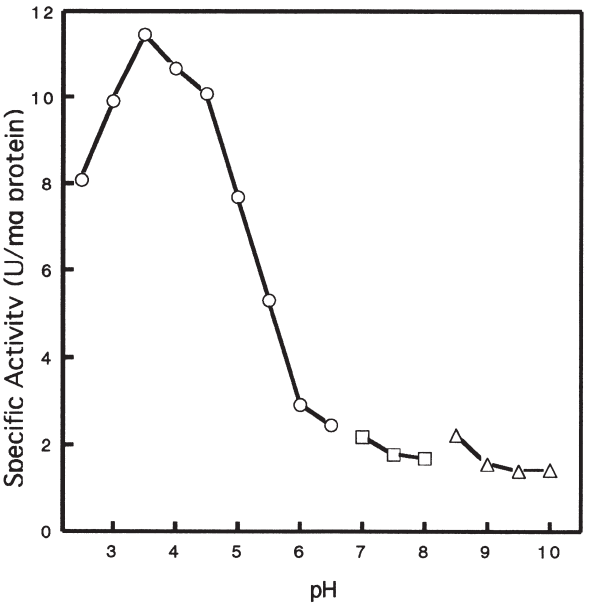

Fig. 1. Effect of pH on hemoglobin hydrolysis by *H. postica* larval midgut homogenate. Buffers were citrate–phosphate (○), phosphate (□), or glycine–NaOH (△). The experiment was repeated twice in replicates of two and the average specific activity of a single experiment graphed. Standard errors were less than 5% of the average for all treatments.

Since one of the most important diagnostic features of cysteine proteinases is enhanced activity in the presence of thiol compounds (Storey and Wagner, 1986), we examined the effect of 1 mM or 5 mM dithiothreitol (DTT), 2-mercaptoethanol (2-ME), or L-cysteine at pH 3.5 and pH 5.0 (Table 1). Considerable activation occurred among all treatments at pH 5.0 with azocasein as substrate, with the highest level of activation (3.3-fold

Table 1
Effect of thiol activators on *H. postica* midgut proteinase activity

Activator[b]		Fold-increase in activity[a]	
		pH 3.5	pH 5.0
Dithiothreitol	1 mM	1.03±0.08	2.11±0.06
	5 mM	1.11±0.04	1.88±0.02
2-Mercaptoethanol	1 mM	0.85±0.02	1.68±0.07
	5 mM	0.78±0.02	1.83±0.06
L-cysteine	1 mM	1.13±0.04	1.85±0.03
	5 mM	1.20±0.04	3.30±0.35

[a] Activity values are given as fold-increase compared to control without activator ±SE derived from averaging the results of two separate experiments. Hemoglobin or azocasein was used as substrate at pH 3.5 and 5.0, respectively.

[b] Reaction mixtures consisted of midgut extract, citrate–phosphate buffer at given pH, 1 mM EDTA, 0.1% Triton X-100, 0.028% (w/v) BSA, 1 mM or 5 mM activator, and 0.67% (w/v) hemoglobin or 1.33% (w/v) azocasein in the final 120 μl reaction volume.

increase) occurring in the presence of 5 mM L-cysteine. Little activation, or even inhibition in the case of 2-ME, was evident at pH 3.5 with hemoglobin as substrate. Inhibition of aspartyl proteinases, such as cathepsin D, by thiol compounds has been reported previously (Barrett, 1977b; Thie and Houseman, 1990), and may explain the inhibition by 2-ME.

3.2. Class-specific inhibition of proteolytic activity

The presence of aspartyl and cysteine proteinases in weevil midgut homogenates was further examined at pH 3.5 and 5.0 by adding low-molecular weight inhibitors known to specifically inhibit these proteinases. Pepstatin A (PEP) and E-64 are known to be largely specific towards aspartyl (Barrett, 1977b) and cysteine proteinases (Hanada et al., 1978; Barrett et al., 1982), respectively. The inhibition pattern obtained with these inhibitors without added thiol activator is presented in Fig. 2A. At pH 3.5, PEP and E-64 treatment resulted in a reduction in total hemoglobinolytic activity of about 32 and 33%, respectively, vs reductions in azocaseinolytic activity of approximately 50 and 42% for the respective inhibitors at pH 5.0. Adding both inhibitors simultaneously to the reaction mixture indicates that aspartyl and cysteine proteinases together account for approximately 80% of the total midgut activity at either pH. PMSF, a serine PI (Barrett, 1977a), did inhibit the extract by as much as 20–30% when tested at pH 5.0 (data not shown); however, this pH is inconsistent with the expected activity range of serine proteinases, and PMSF is known to inhibit cysteine proteinases (Storey and Wagner, 1986) in the absence of reductants. Furthermore, an inhibitor of both cysteine and serine proteinases (leupeptin) (Barrett and Kirschke, 1981) gave a similar pattern and level of inhibition as E-64 at concentrations ranging from 0.1–200 μM (data not shown). This is inconsistent with a serine-like proteolytic activity, as leupeptin would be expected to exceed the inhibition of E-64 if both classes of proteinases were present in the extract. The metalloproteinase inhibitor EDTA (Barrett, 1977a) did not inhibit proteolytic activity at either pH when included at 1 mM or 5 mM (data not shown). Inhibition experiments were not conducted at higher pH values due to the absence of measurable hemoglobinolytic or azocaseinolytic activity at pH ≥7.

The absolute level of inhibition obtained with each treatment varied with several factors that included the concentration of inhibitor, duration of extract-inhibitor co-incubation prior to substrate addition, presence or absence of reducing agent in the reaction, and the type of substrate (data not shown). Both pepstatin A and E-64 were highly potent, exerting most of their effect at concentrations below 1.0 μM. Although co-incubation of inhibitor and extract prior to addition of the substrate generally resulted in more inhibition, it was necessary to limit the duration of co-incubation and include BSA in the reaction in order to minimize auto-degradation of proteolytic activity in the sample. Consequently, the maximum level of inhibition reported is not necessarily the maximum level of inhibition achievable. Inclusion of reducing agent resulted in a relative shift in the pro-

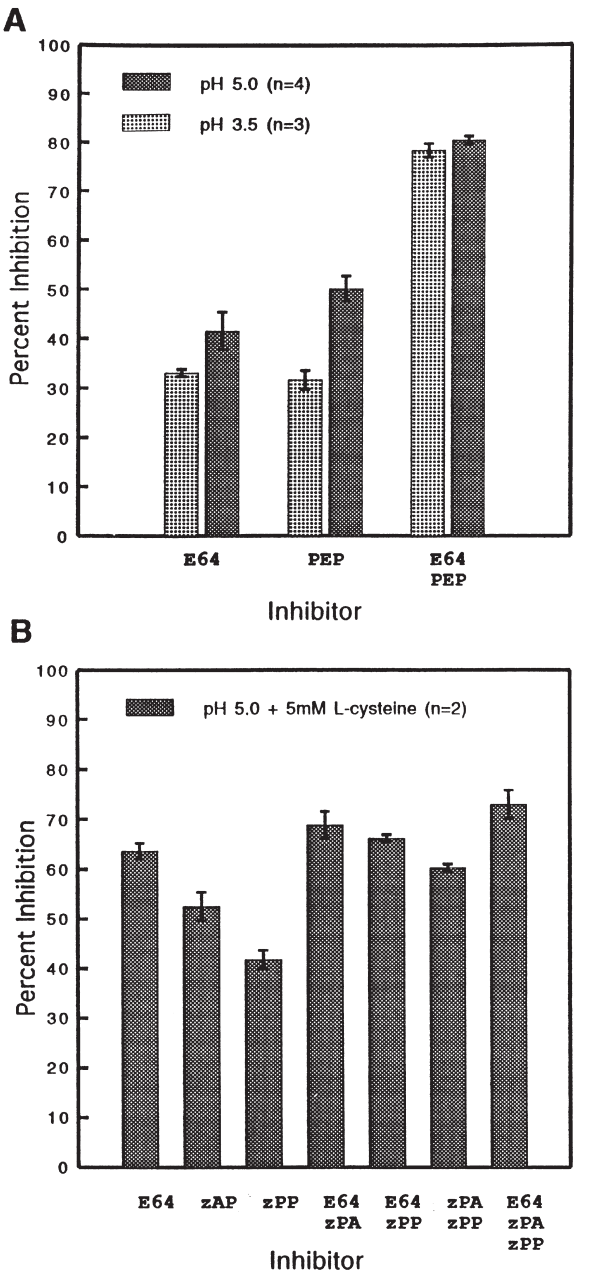

Fig. 2. Inhibition of proteolytic activity in larval midgut homogenates. (A) Inhibition of proteinases by class-specific proteinase inhibitors at both pH 3.5 and pH 5.0. (B) Inhibition of proteinases with diazomethyl ketone inhibitors at pH 5.0 in the presence of 5 mM L-cysteine. E64, L-*trans*-Epoxysuccinyl–leucylamido(4-guanidino) butane; PEP, pepstatin A; zPA, Z-Phe–Ala–CHN_2; zPP, Z-Phe–Phe–CHN_2. All inhibitors were used in excess at 100 μM with hemoglobin (pH 3.5) or azocasein (pH 5.0) provided as substrate. Experiments were repeated *n* times and the average percent inhibition ±SE is presented.

minence of cysteine proteinases in the extract, as reflected by comparatively higher levels of E-64 inhibition. This effect, however, was much more prominent when azocasein was supplied as the substrate, as thiol activation was substantially less with hemoglobin.

The nature of the cysteine proteinase fraction of activity belonging to the crude extract was examined by employing two irreversible diazomethyl ketone inhibitors specific to the cysteine proteinase cathepsins B and L (Shaw and Green, 1981; Riemann et al., 1982). Z-Phe–Ala–CHN_2 (zPA) and Z-Phe–Phe–CHN_2 (zPP) are both very effective inhibitors of cathepsin L with apparent k_{2nd} reaction rates of 70,000 $M^{-1}\cdot s^{-1}$ and 160,000 $M^{-1}\cdot s^{-1}$, respectively. However, zPA is a much better inhibitor of cathepsin B (k_{2nd} ranging from 500–2000 $M^{-1}\cdot s^{-1}$) than is zPP (k_{2nd} about 200 $M^{-1}\cdot s^{-1}$) (Barrett and Kirschke, 1981). Neither inhibitor is effective against cathepsin H cysteine proteinase. The effect of these inhibitors on thiol-activated extract in comparison to, or in combination with, the broad cysteine PI E-64 (96,250 $M^{-1}\cdot s^{-1}$, 89,400 $M^{-1}\cdot s^{-1}$, and 4000 $M^{-1}\cdot s^{-1}$ for cathepsins L, B, and H, respectively) (Barrett and Kirschke, 1981) is presented in Fig. 2B. The cathepsin L-specific inhibitor, zPP, inhibited about 42% of the crude azocaseinolytic activity as compared to 64% inhibition by E-64. The inhibitor of B and L cathepsins, zPA, inhibited approximately 53% of the azocaseinolytic activity. As expected, the combination of a diazomethyl ketone inhibitor with E-64 did not result in any sizable increase in inhibition as compared to E-64 alone, and the level of inhibition obtained from the combination of zPA and zPP (ca. 60%) approximated the level of E-64 inhibition.

3.3. Inhibition by protein inhibitors

The relative effect of several plant-derived protein inhibitors on midgut extract activity is presented in Fig. 3. The soybean trypsin/chymotrypsin inhibitor (Bowman-Birk inhibitor I, or BBI) is a known inhibitor of many serine proteinases. API inhibits cathepsin D-like aspartyl proteinases as well as certain serine proteinases, such as trypsin and chymotrypsin, but not pepsin-like aspartyl proteinases (Keilova and Tomasek, 1976a). Rice cysteine PI oryzacystatin I (OCI) effectively inhibits cathepsins H and L, but not B (Abe et al., 1994). API and OCI (as a GST fusion protein) were highly effective against the extract. At pH 3.5, API and OCI treatments resulted in an approximate reduction in total hemoglobinolytic activity of 40 and 26%, respectively, vs reductions in azocaseinolytic activity of 51 and 34% for the respective inhibitors at pH 5.0. These inhibitors were additive when used in combination, resulting in about 68 and 73% inhibition at pH 3.5 and 5.0, respectively, suggesting that they target distinct proteinase components in the extract. As expected, BBI was largely

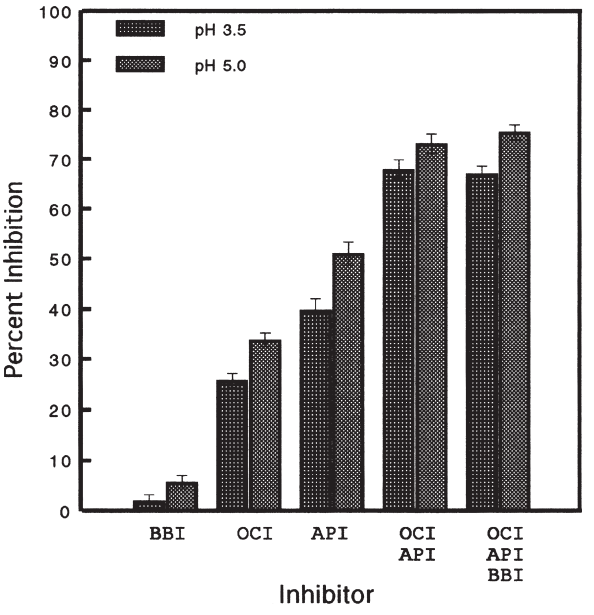

Fig. 3. Inhibition of proteolytic activity in larval midgut homogenates by plant-derived PIs. BBI, Bowman-Birk inhibitor I (soybean trypsin–chymotrypsin inhibitor); OCI, oryzacystatin I; API, aspartyl proteinase inhibitor. Test levels were 50 μg for OCI and BBI, and 1.4 μg for API. Each reaction consisted of 6–14 μg crude midgut protein with hemoglobin (pH 3.5) or azocasein (pH 5.0) provided as substrate. Experiments were repeated three times and the average percent inhibition ±SE is presented.

ineffective against the crude proteolytic activity of the extract. These results are consistent with the inhibition data obtained using the low-molecular weight inhibitors (Fig. 2) of cysteine and aspartyl proteinases.

Although Fig. 3 represents inhibition levels where the individual inhibitors are supplied at above saturation levels, both API and OCI were rather potent inhibitors based upon the concentration of inhibitor that resulted in approximately 50% of their effect (IC_{50}) (data not shown). Their IC_{50} was approximately 0.1 μg GST-OCI and 0.002 μg API for each microgram of crude alfalfa weevil midgut protein in the reaction. Adjusting for the molecular weight of OCI (12,000 Da) in comparison to the total size of the GST fusion (43,000 Da), this amounts to 0.03 μg OCI for each microgram of midgut protein. By comparison, the related rice cysteine PI oryzacystatin II (Kondo et al., 1990) that strongly inhibits cathepsin H but is not as good an inhibitor of cathepsins L and B as OCI (Abe et al., 1994), inhibited the extract at levels equivalent to OCI when supplied at ≥50 μg, but had an IC_{50} that was more than 30-fold higher (1 μg OCII per μg midgut protein).

4. Discussion

The primary aim of this work was to identify the major digestive proteinases of *H. postica*. In a previous

survey of Coleoptera insects by Murdock et al. (1987), *H. postica* was shown to possess proteolytic activity consistent with the action of cysteine proteinases. Methemoglobin hydrolysis by midgut homogenates at pH 5.5 was stimulated by the addition of 5 mM L-cysteine and inhibited by the alkylating agent *p*CMB. Elden (1995b) subsequently determined that several low-molecular weight inhibitors of cysteine proteinases (E-64, *p*HMB, and leupeptin) adversely affected alfalfa weevil growth and development when ingested in an in vivo bioassay. These inhibitors also reduced foliar feeding by alfalfa weevil larvae, as did the API pepstatin A, but to a lesser degree than the cysteine PIs. The present study presents a more detailed description of the digestive proteinases of *H. postica*, and identifies corresponding plant-derived inhibitors with potential use in transgenic applications.

Alfalfa weevil midguts contain a high level of proteolytic activity on general substrate up to about pH 6 in the absence of reducing agents (Fig. 1). That this activity exists in the acidic to mildly-acidic pH range, and is largely blocked by the addition of inhibitors of aspartyl and cysteine proteinases (Fig. 2A), indicates that these two proteinase classes contribute the majority of proteolytic activity to the extracts. Although each class is inhibited at levels similar to one-another at the test pH values of 3.5 and 5.0 *in vitro*, their physiological importance may differ. The luminal content of alfalfa weevil midguts has been determined previously to have a pH of 6.6 (Elden, 1995b). Noting that this value disregards the spatial differences in pH that likely exist in intact insect midguts (Terra and Ferreira, 1994), and thus does not rule out the action of aspartyl proteinases, this pH is more consistent with the action of cysteine proteinases. Furthermore, the fraction of total proteolytic activity due to cysteine proteinases was substantially increased when reducing agent was included in the reaction (Fig. 2B). This suggests that cysteine proteinase activity could be substantial in midguts with a negative (reducing) redox potential. We speculate that the redox potential of the alfalfa weevil is reducing, as is the case of other Coleoptera beetle species believed to utilize cysteine proteinases as digestive enzymes (Murdock et al., 1987). The results of the in vivo bioassays (Elden, 1995b) using inhibitors of cysteine and aspartyl proteinases (discussed above) further support the conclusion that the cysteine class is likely the more physiologically-relevant in this insect and that the aspartyl class may have a secondary role.

Cathepsin L appears to be the major cysteine proteinase in larval midguts of the weevil. A cathepsin L-specific inhibitor, Z-Phe–Phe–CHN_2, blocked approximately two-thirds of the total cysteine proteolytic activity (as measured by E-64 inhibition) in thiol-activated extracts (Fig. 2B). This is supported by the fact that a random screen of 10 clones from a cDNA library prepared using midgut-specific mRNA yielded a cDNA insert encoding a cathepsin L enzyme (GenBank Accession # AF157961) (Wilhite and Smigocki, unpublished). Much of the remaining one-third of cysteine proteinase activity associated with the extract is consistent with the action of cathepsin B. This is evident in the enhanced inhibition obtained with Z-Phe–Ala–CHN_2 compared to Z-Phe–Phe–CHN_2, as well as the fact that, when combined, they approach the level of inhibition obtained with the broad cysteine PI E-64. In addition, leupeptin, which inhibits cathepsins B and L, but not H (Barrett and Kirschke, 1981), is also similar to E-64 in potency. These results support a major role for cathepsin L, a minor role for cathepsin B, and little or no role for cathepsin H. On this basis, OCI would be expected to target the major cysteine proteinase component in the weevil extract since this phytocystatin strongly inhibits cathepsin L; however, cathepsin B is not inhibited by OCI (Abe et al., 1994). This is consistent with the relationship we typically observe between OCI and E-64, as OCI inhibition is usually about two-thirds to three-quarters that of E-64. Inefficient inhibition by OCII (data not shown, see Results), which has weak inhibitory activity toward cathepsin B (K_i=8.2×10^{-6}), is likely due to its decreased affinity for cathepsin L (K_i=3.9×10^{-8}) in comparison to OCI (K_i=5.1×10^{-9}) (Abe et al., 1994). A more reasonable choice would be an inhibitor with potency against both cathepsin B and L, such as the recently characterized kidney bean phytocystatin, FSCPI 5.5 (Brzin et al., 1998).

The API preparation that we used in this study consists of several isoforms of potato cathepsin D inhibitor (PDI) that is structurally related to the soybean trypsin inhibitor family (Mares et al., 1989). It inhibits cathepsin D as well as the serine proteinases trypsin and chymotrypsin, but does not affect pepsin activity (Keilova and Tomasek, 1976a). That this inhibitor preparation was equal in effectiveness to that of pepstatin A, which inhibits pepsin as well as cathepsin D, suggests that cathepsin D is the major aspartyl proteinase in the extract. This conclusion is strengthened by the fact that a preparation of squash aspartic proteinase inhibitor (SQAPI), which inhibits pepsin but not cathepsin D (Christeller et al., 1998), had no effect on the extract (data not shown). Whether PDI will be useful in transgenic applications will hinge in large part on the effectiveness of the individual isoforms, as expressed from their cDNAs, on proteolytic activity. In addition, we have not studied the compartmentalization of enzymatic activity, so it is unknown if the ingested inhibitor would reach its target enzyme following ingestion. It is conceivable that this enzyme has a function other than initial digestion in the endoperitrophic space, as is generally true of insect midgut endoproteinases (Terra and Ferreira, 1994). Cathepsin D from rat is capable of activating cathepsin B by proteolytic cleavage of the propeptide to produce the mature form of the enzyme (Rowan et al., 1992). Zymogen activation of cathepsins L and B by

cathepsin D here would likely place this enzyme within another region of the digestive system, such as within the midgut cells themselves (Terra and Ferreira, 1994). In this case, it seems unlikely that ingested PDI would have any effect on insect growth and development.

The present study has identified cathepsins L, B, and D as the major proteinase forms existing in *H. postica* midguts. Thus, any rational approach of insect control in transgenic plants expressing PI genes should target each of these digestive proteinases. The necessity of such a strategy has become increasingly evident with the understanding that many insects possess very effective resistance mechanisms against individual PIs. Resistance has been attributed to complex proteolytic systems, allowing the insects to degrade PIs in Coleoptera (Girard et al., 1998a) and Lepidoptera (Giri et al., 1998), and to enhance the production of inhibitor-insensitive proteinases in response to PI ingestion in Coleoptera (Girard et al., 1998b; Bonade-Bottino et al., 1999; Cloutier et al., 1999) and Lepidoptera (Broadway 1995, 1997; Jongsma et al., 1995; Broadway, 1996b; Brown et al., 1997; Wu et al., 1997). It has been suggested (Orr et al., 1994; Broadway, 1996a) that such complex mechanisms are most likely to exist in polyphagous insects having generalized feeding habits compared to oligo- or monophagous insects. Although we did not examine the issue of inhibitor-insensitivity per se, it seems likely that *H. postica*, which is oligophagous, has a proteolytic system complex enough to necessitate a defense strategy involving the co-expression of multiple PIs. A cysteine PI highly potent against cathepsins L and B should be used in combination with an aspartyl PI, such as PDI. The ability of PDI to inhibit serine proteinases is an attractive feature since these proteinases could possibly exist in the proteolytic arsenal of *H. postica*, even though we did not detect them here, or be utilized by other insect pests of alfalfa.

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