

Digestive proteinases in *Lasioderma serricorne* (Coleoptera: Anobiidae)

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Abstract

The cigarette beetle, *Lasioderma serricorne* (Fabricius), is a common pest of stored foods. A study of digestive proteinases in *L. serricorne* was performed to identify potential targets for proteinaceous biopesticides, such as proteinase inhibitors. Optimal casein hydrolysis by luminal proteinases of *L. serricorne* was in pH 8.5–9.0 buffers, although the pH of luminal contents was slightly acidic. Results from substrate and inhibitor analyses indicated that the primary digestive proteinases were serine proteinases. The most effective inhibitors of caseinolytic hydrolysis were from soybean (both Bowman Birk and Kunitz), with some inhibition by chymostatin, *N*-tosyl-L-phenylalanine chloromethyl ketone, and leupeptin. Casein zymogram analysis identified at least eight proteolytic activities. Activity blot analyses indicated one major proteinase activity that hydrolysed the trypsin substrate *N*- α -benzoyl-L-arginine *p*-nitroanilide, and three major proteinase activities that hydrolysed the chymotrypsin substrate *N*-succinyl ala-ala-pro-phe *p*-nitroanilide. The absence of cysteine, aspartic, and metallo proteinases in *L. serricorne* digestion was evidenced by the lack of activation by thiol reagents, alkaline pH optima, and the results from class-specific proteinase inhibitors. The data suggest that protein digestion in *L. serricorne* is primarily dependent on trypsin- and chymotrypsin-like proteinases.

Introduction

The cigarette beetle, *Lasioderma serricorne* (Fabricius) (Coleoptera: Anobiidae), is a cosmopolitan pest of a wide variety of stored raw commodities, as well as finished food products including spices, seeds, grains, dried potatoes, raisins and tobacco (Richards & Herford, 1930; Howe, 1957; Catterji *et al.*, 1963; LeCato, 1978). Although the common name is a consequence of the serious economic injury to stored tobacco, *L. serricorne* has a more diverse food selection than most other stored product pests and has been documented attacking over 50 different plant and animal sources (Howe, 1957). *Lasioderma serricorne* has also been found in household drugs, dried fish and dried plant material such as flower arrangements and book bindings.

Fumigants have been used to control *L. serricorne* (Zettler & Arthur, 2000). However, control failures with phosphine

have been attributed to resistance development (Rajendran & Narasimhan, 1994). The inclusion of biological control agents into the integrated pest management of *L. serricorne* has been suggested as a method of achieving more effective, long-term control (Eberhardt, 1997). In addition, the insecticidal proteins from *Bacillus thuringiensis* Berliner (Bacillaceae) have been proposed as a potential biopesticide for *L. serricorne* control (Kaelin *et al.*, 1999). The long-term goal of our research is to identify biopesticides that target digestion for incorporation into an integrated pest management programme for *L. serricorne*.

Lasioderma serricorne is a phytophagous insect in the Anobiidae family, a group that includes other species capable of digesting dried plant and wood products. Serine proteinases have been described in the larger grain borer, *Prostephanus truncatus* (Horn) and lesser grain borer, *Rhyzopertha dominica* (Fabricius), that belong to the closely related family Bostrichidae (Houseman & Thie, 1993; Zhu & Baker, 1999). However, little is known about digestive proteinases in species belonging to the Anobiidae. In an

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effort to design cereals with enhanced resistance to *L. serricornis*, and part of a larger project to define digestive proteinases in coleopteran pests of stored products, class-specific substrates and inhibitors were used to partially characterize digestive proteinases in *L. serricornis*.

Materials and methods

Insect dissection and pH measurement

Larvae were obtained from a laboratory colony of *L. serricornis* reared on 95% wheat flour and 5% brewer's yeast, compacted and top-layered with cracked wheat. Larvae weighed an average of $3.2 \text{ mg} \pm 0.1$ (mean \pm S.E., $n = 9$ groups of 10 each) and had an average head capsule size of $0.59 \text{ mm} \pm 0.01$ ($n = 4$ groups of 10 each). For proteinase assays, larvae were chilled on ice, and lumens were extracted following removal of the posterior and anterior ends and immediately placed in $25 \mu\text{l}$ of ice-cold buffer A (200 mM Tris-HCl, pH 8.0, 20 mM CaCl) per 10 guts, and frozen at -20°C . Samples were thawed, vortexed briefly, and centrifuged at $15,000 \times g$ for 5 min, and the supernatant was used for assays.

To estimate the pH of luminal contents, lumens were extracted into deionized water (10 guts per $50 \mu\text{l}$), vortexed, and pH was assessed using either a semi-micro pH electrode (Lazar, Los Angeles, California) or pH indicator strips (Baxter Healthcare Corporation, McGaw Park, Illinois).

Microplate proteinase assays

pH optima

The procedure was adapted from a microplate assay (Oppert *et al.*, 1997) as modified by Oppert *et al.* (2000). For pH curves, 0.8 gut equivalents were diluted into $90 \mu\text{l}$ of buffers of various pH, obtained using a universal buffering system (Frugoni, 1957). To evaluate the hydrolysis of a general proteinase substrate, $0.1 \mu\text{g}$ fluorescently-labelled casein (BODIPY-TR-X casein, Molecular Probes, Eugene, Oregon) in $10 \mu\text{l}$ of water was added to each well to initiate the reaction. Samples were incubated at 37°C , and the fluorescence was measured (excitation 584, emission 620) and corrected by subtracting readings obtained with incubations of substrate only (no enzyme). Measurements of enzyme and buffer or buffer alone produced negligible fluorescence (data not shown).

Substrate analyses

Class-specific substrates were obtained from Sigma Chemical Co. (St Louis, Missouri) and included *N*- α -benzoyl-L-arginine *p*-nitroanilide (a trypsin substrate) and *N*-succinyl ala-ala-pro-phe *p*-nitroanilide (a chymotrypsin substrate). Wells contained triplicates of 0.8 gut equivalents in $50 \mu\text{l}$ of universal buffers of various pH. Substrates in the same buffer were added to the appropriate well and incubated at 37°C for 5 min ($10 \mu\text{g}$ per $50 \mu\text{l}$; final concentrations of *N*- α -benzoyl-L-arginine *p*-nitroanilide and *N*-succinyl ala-ala-pro-phe *p*-nitroanilide were 2.30 and 1.60 mM, respectively). Absorbance was read at 405 nm at 15 s intervals, and autohydrolysis of the substrate was corrected by subtracting readings of substrate alone. The change in absorbance per min was calculated by the software KineticCalc3 (Bio-Tek, Winooski, Vermont). Specific activity

was calculated as the number of μmoles of nitroaniline released per min per gut equivalent (using an extinction coefficient of $8700 \text{ M}^{-1} \text{ cm}^{-1}$, determined experimentally for $100 \mu\text{l}$ volumes).

Inhibitor analyses

Inhibitors/activators used in this study were: L-cysteine, chymostatin, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane, ethylenediamine tetra-acetic acid, phenylmethylsulphonyl fluoride, soybean trypsin-chymotrypsin (Bowman Birk) inhibitor, soybean trypsin (Kunitz) inhibitor, *N*- α -*p*-tosyl-L-lysine chloromethyl ketone, and *N*-tosyl-L-phenylalanine chloromethyl ketone, all obtained from Sigma; leupeptin and pepstatin were obtained from Roche Molecular Biochemicals (Indianapolis, Indiana). Inhibitors/activators were prepared as stock solutions and were serially diluted in $50 \mu\text{l}$ of universal buffer, pH 9.2, in microplate wells. To each well, 0.4 gut equivalents of *L. serricornis* luminal extract in $40 \mu\text{l}$ of universal buffer, pH 9.2, were added. Microplates were incubated at 37°C for 15 min prior to the addition of substrate (BODIPY). The remainder of the procedure was followed as described in the pH optima experiment. The concentrations resulting in 50% inhibition of non-inhibited enzymes (IC_{50}) were calculated using linear regression.

Zymogram analysis

Aliquots of *L. serricornis* luminal contents (1.6 gut equivalent per lane) were separated by non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 4–16% zymogram blue casein gels (ZBC, Invitrogen, San Diego, California). Gels were developed in universal buffer, pH 6.3 or 11.2, \pm L-cysteine for 2 h, at 37°C . Clear zones indicated active proteinases.

Proteinase activity blots

Lasioderma serricornis luminal extracts (2 gut equivalents per lane) were subjected to SDS-PAGE using 10–20% tricine gels and MultiMark molecular mass standards (Invitrogen). Proteins were electrotransferred to nitrocellulose and incubated with *p*-nitroanilide substrates in buffer A and developed by subsequent incubations of 5 min each in 0.1% sodium nitrite in 1 M hydrochloric acid, 0.5% ammonium sulphamate in 1 M hydrochloric acid, and 0.05% *N*-(1-naphthyl)-ethylenediamine in 47.5% ethanol, as described previously (Oppert & Kramer, 1998).

Results

The pH of luminal extracts from *L. serricornis* larvae was 6.5 ± 0.03 (mean \pm S.E., $n = 3$) when measured with a microelectrode, and a value of 6.3 was estimated with pH indicator strips. In contrast to the weakly acidic pH in the lumen, the optimal pH values for hydrolysis of casein by *L. serricornis* luminal proteinases were in the alkaline range, from 8.5–9.2 (fig. 1). Casein hydrolysis was observed in buffers ranging from pH 5.3–11.7.

Hydrolysis of *p*-nitroanilide substrates by *L. serricornis* luminal proteinases was observed over a broad pH range (fig. 2). Hydrolysis of *N*- α -benzoyl-L-arginine *p*-nitroanilide occurred in alkaline buffers from pH 9.2–11.7, with an optimum at 10.6. Hydrolysis of *N*-succinyl ala-ala-pro-phe *p*-

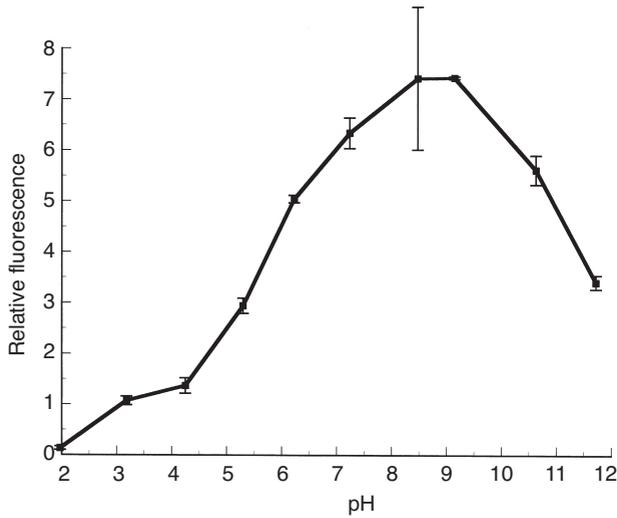


Fig. 1. Hydrolysis of fluorescent casein by luminal extracts from *Lasioderma serricorne* in buffers of different pH, following a 2 h incubation.

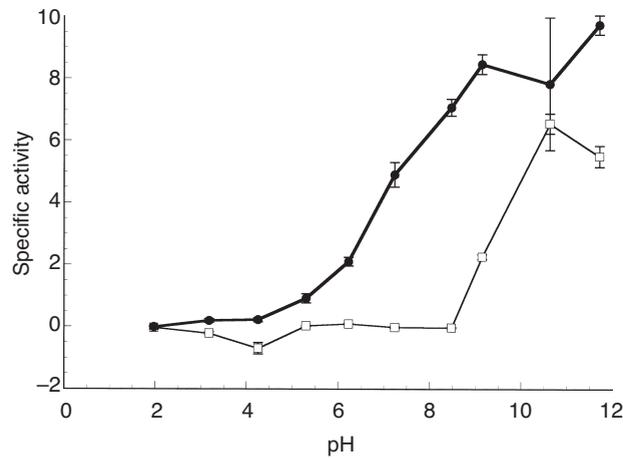


Fig. 2. pH dependence on the hydrolysis of substrates *N*- α -benzoyl-L-arginine ρ -nitroanilide (□) and *N*-succinyl ala-ala-pro-phe ρ -nitroanilide (●) by luminal extracts from *Lasioderma serricorne*. Specific activity was calculated as the number of μ moles of nitroaniline released per min per gut equivalent using an extinction coefficient of $8700 \text{ M}^{-1} \text{ cm}^{-1}$ determined for $100 \mu\text{l}$ volumes.

nitroanilide occurred in acidic buffers of pH 5.3 and increased concomitantly with the increase in the alkalinity of the buffer.

Proteinase activity blot analyses demonstrated the presence of multiple proteinase activities (fig. 3a). Activity blots with *N*-succinyl ala-ala-pro-phe ρ -nitroanilide as substrate presented three major bands of activities (C1, C2 and C3), corresponding to proteins with molecular mass approximately >100 , $70\text{--}90$ and 20 kDa , respectively (fig. 3a, left panel). The broad band of activity may be indicative of multiple proteinase activities within each band. In activity blots with the substrate *N*- α -benzoyl-L-arginine ρ -nitroanilide,

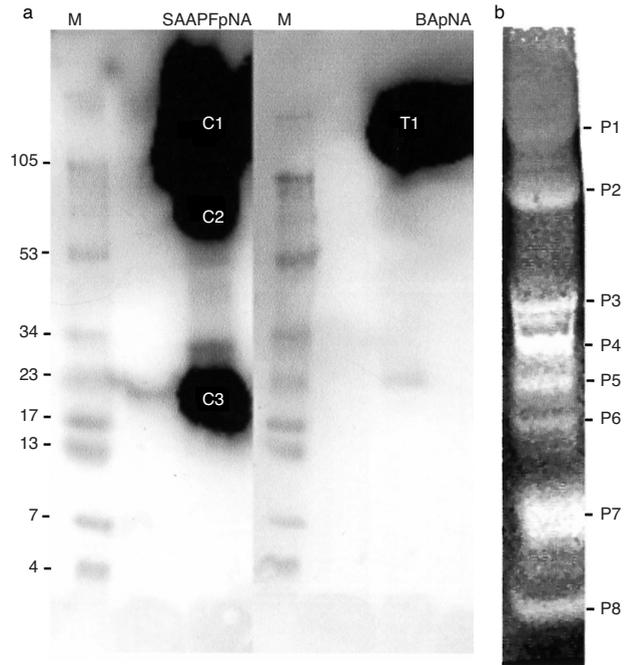


Fig. 3. Proteinase activity in luminal extracts from *Lasioderma serricorne*: (a) activity blot using the substrates *N*-succinyl ala-ala-pro-phe ρ -nitroanilide (SAAPFPNA) and *N*- α -benzoyl-L-arginine ρ -nitroanilide (BAPNA). C1, C2 and C3 refer to chymotrypsin-like proteinases. T1 refers to trypsin-like proteinases. M = molecular mass standards; (b) casein zymogram. P1–P8 refer to general caseinolytic proteinase activities.

one major band of proteinase activity was observed (T1), corresponding to proteins with molecular masses approximately in the range of $100\text{--}200 \text{ kDa}$ (fig. 3a, right panel).

In the casein-zymogram analysis, at least eight different caseinolytic activities (P1–P8) were observed (fig. 3b). These activities were observed at similar intensities with or without thiol reagents (L-cysteine) in either buffer pH 6.3 or 11.2 (data shown for pH 6.3, without L-cysteine zymogram). Because the presence of casein in the gel affects the migration of some proteins, no correlation was made between proteinases hydrolysing *N*- α -benzoyl-L-arginine ρ -nitroanilide, *N*-succinyl ala-ala-pro-phe ρ -nitroanilide and casein. There was also a difference in the gel systems used. The activity blots were performed with 10–20% gradient tricine gels, whereas the zymograms were in 4–16% tris-glycine gels containing casein.

The most potent inhibitors of *L. serricorne* luminal extract caseinolytic activity were the soybean inhibitors, soybean trypsin-chymotrypsin (Bowman Birk) inhibitor and soybean trypsin (Kunitz) inhibitor (fig. 4). Chymostatin and *N*-tosyl-L-phenylalanine chloromethyl ketone moderately inhibited caseinolytic activity by *L. serricorne* proteinases, and leupeptin demonstrated slight inhibition in the range tested. Phenylmethylsulphonyl fluoride was only inhibitory at higher (mM) concentrations. IC_{50} values for soybean trypsin-chymotrypsin (Bowman Birk) inhibitor and soybean trypsin (Kunitz) inhibitor were 0.21 and $1.28 \mu\text{M}$, respectively (table 1). The IC_{50} for chymostatin was $24 \mu\text{M}$, while the IC_{50} values were 18.4 mM for *N*-tosyl-L-phenylalanine chloromethyl

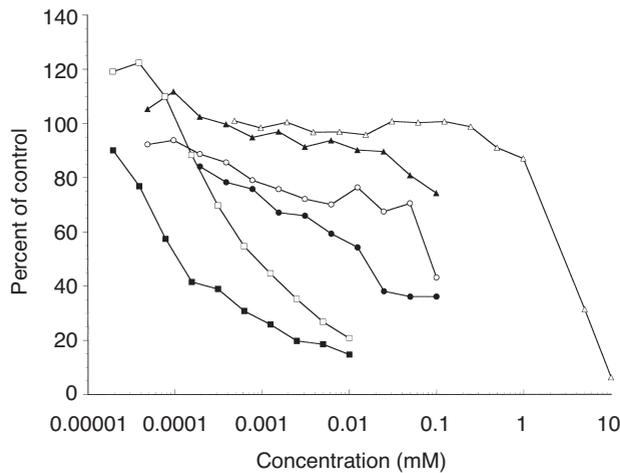


Fig. 4. Inhibition of *Lasioderma serricorne* luminal proteinases by selected inhibitors, as a percentage of the uninhibited (control) activity. ■, soybean trypsin-chymotrypsin (Bowman Birk) inhibitor; □, soybean trypsin (Kunitz) inhibitor; ●, chymostatin; ○, *N*-tosyl-L-phenylalanine chloromethyl ketone; ▲, leupeptin; △, phenylmethylsulphonyl fluoride.

ketone, 3.65 mM for phenylmethylsulphonyl fluoride, and 16.9 M for leupeptin. Inhibitors with no effect on caseinolytic activity included concentrations up to 10 mM ethylenediamine tetra-acetic acid, 0.2 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane, 0.1 mM *N*- α -p-tosyl-L-lysine chloromethyl ketone, and 0.1 mM pepstatin (data not shown). In addition, L-cysteine at concentrations up to 10 mM did not stimulate caseinolytic activity (data not shown).

Discussion

Our data demonstrated that *L. serricorne* digests protein via serine proteinases. The evidence for this hypothesis includes alkaline pH optima for the hydrolysis of general proteinase substrates, hydrolysis of trypsin and chymotrypsin substrates, and specific inhibition by serine proteinase inhibitors, especially those from soybean. Genes from soybean encoding serine proteinase inhibitors may therefore be good candidates for the development of transgenic seed for enhanced protection against *L. serricorne* damage during storage. Activity blot analyses suggested that many *L. serricorne* luminal proteinases have chymotrypsin-like properties. Therefore, specific inhibitors of insect chymotrypsins may be particularly effective.

Although the pH of *L. serricorne* luminal contents was slightly acidic, casein hydrolysis was highest in alkaline buffer. Differences in values of the luminal pH and optimal pH for casein hydrolysis may be attributed to several factors. Gradients in pH have been documented within the insect lumen, and proteinases are often localized to specific regions (Berenbaum, 1980). Alternatively, the luminal extract contains a mixture of proteins, and the properties of isolated proteinases may differ. Proteinases are active over a range of pH values and may be more stable at pH values other than their respective maxima.

Hydrolysis of *p*-nitroanilide substrates by *L. serricorne* luminal proteinases in alkaline buffers suggests the presence of serine proteinases. The hydrolysis in slightly acidic buffers of *N*-succinyl ala-ala-pro-phe *p*-nitroanilide, typically a chymotrypsin substrate, could be due to cysteine proteinase activity, found in some coleopteran insects. However, inhibition of *L. serricorne* caseinolytic activity was not observed with cysteine proteinase inhibitors. Cysteine

Table 1. Relative inhibition of *Lasioderma serricorne* luminal proteinases by selected inhibitors *in vitro*.

Inhibitor	Target proteinase(s)	Concentration tested (mM)	IC ₅₀ ¹ (μ M)
Soybean trypsin-chymotrypsin (Bowman Birk) inhibitor	² Trypsin and chymotrypsin	0.00002–0.01	0.21 (0.04–1.28)
Soybean trypsin (Kunitz) inhibitor	³ Trypsin, factor Xa, plasmin, and plasma kallikrein	0.00002–0.01	1.28 (0.48–3.40)
Chymostatin	³ α -, β -, γ -, δ -chymotrypsin	0.002–0.1	24.0 (12.0–47.8)
<i>N</i> -tosyl-L-phenylalanine chloromethyl ketone	³ Irreversible inhibitor of chymotrypsin, and inhibits other serine and cysteine proteases such as bromelain, ficin, and papain	0.0001–0.1	1.84 $\times 10^3$ (120–2.83 $\times 10^3$)
Phenylmethylsulphonyl fluoride	³ Serine proteases and some cysteine proteases	0.0005–10.0	3.65 $\times 10^3$ (0.77–17.3 $\times 10^3$)
Leupeptin	³ Serine and cysteine proteases, such as trypsin, papain, plasmin, and cathepsin B	0.0001–0.1	1.69 $\times 10^7$ (2.74 $\times 10^5$ –1.03 $\times 10^9$)
<i>N</i> - α -p-tosyl-L-lysine chloromethyl ketone	³ Irreversible inhibitor of trypsin, and inhibits other serine and cysteine proteases such as bromelain, ficin, and papain	0.0001–0.1	⁴ NI
Trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane	³ Papain and other cysteine proteases like cathepsin B and L	0.0001–0.2	NI
Ethylenediamine tetra-acetic acid	³ Metalloproteases	0.02–10.0	NI

¹Concentration resulting in 50% inhibition of the uninhibited activity (95% C.I. in parentheses). ²(Birk, 1985). ³As per Boehringer Mannheim Proteinase Inhibitors Technical Guide (<http://biochem.roche.com>).

⁴NI, no inhibition in the concentration range tested.

proteinase activity is generally enhanced in the presence of thiol reagents, and no increases were observed in the hydrolysis of casein with buffers containing thiol reagent in the zymogram analysis. Furthermore, similar banding patterns were observed in casein zymograms incubated in acidic or alkaline buffers. These data suggest that *L. serricorne* serine proteinases with chymotrypsin-like activity are active in buffers of broader pH range than trypsin-like proteinases, which apparently are only active in alkaline buffers.

At least eight major proteinase activities were detected in the luminal extracts from *L. serricorne* using zymogram analysis. The activity blot analyses indicated that at least some of these activities contributed to the hydrolysis of trypsin and chymotrypsin substrates. Whether T1, the largest molecular mass activity with trypsin-like characteristics, is the same proteinase as C1, the largest molecular mass chymotrypsin-like activity, remains to be determined. However, slower migrating proteinases (P1 and P2) may be the same as C1, C2 and T1 activities. The fastest migrating chymotrypsin activity, C3, is likely one of the proteinases P3-P8. However, the range in molecular masses of proteinases P3-P8 would suggest that proteinases other than those capable of hydrolysing the synthetic trypsin and chymotrypsin substrates may be contributing to protein digestion.

Broad bands of activity of large molecular mass proteinases occurred in zymograms and proteinase activity blots with both ρ -nitroanilide substrates. Large molecular mass proteinase activities have been observed in luminal extracts from the Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Oppert *et al.*, 1996). One explanation for these large molecular mass proteinase activities may be proteinases that are postulated to remain associated with a jelly-like material on the peritrophic membrane (Bolognesi *et al.*, 2001). Although electrophoresis is performed under denaturing conditions, these proteinase-lipid complexes may not dissociate under the conditions adopted to retain proteinase activity, namely non-reducing buffers and no heating of samples prior to electrophoresis. Alternatively, these activities may be due to large molecular mass proteinases or proteinase complexes. Purification of both membrane and soluble proteinases and additional experiments with lipases are needed to further address these large molecular mass proteinase activities.

Micromolar concentrations of soybean inhibitors were effective in the inhibition of *L. serricorne* luminal proteinases. However, millimolar concentrations of another serine proteinase inhibitor, phenylmethylsulphonyl fluoride, were needed to achieve similar levels of inhibition. *Lasioderma serricorne* luminal proteinases were inhibited by phenylmethylsulphonyl fluoride concentrations greater than 1 mM, with approximately 95% inhibition observed at 10 mM (data not shown). This same pattern of inhibition by phenylmethylsulphonyl fluoride has been reported for other stored product insect digestive proteinases (Oppert *et al.*, 1996, 2000). Some purified proteinases from other insects are inhibited by phenylmethylsulphonyl fluoride (see <http://bru.usgmr1.ksu.edu/db/proteinases/> for a list of proteinases and supporting references). However, usually these studies tested only single concentrations of phenylmethylsulphonyl fluoride, often in the millimolar range. Purification of *L. serricorne* proteinases will be necessary to determine if isolated proteinases and proteinase mixtures respond to inhibitors differently.

Inhibition of *L. serricorne* proteinases by chymotrypsin inhibitors, including soybean trypsin-chymotrypsin (Bowman Birk) inhibitor, chymostatin and N-tosyl-L-phenylalanine chloromethyl ketone correspond to the hydrolysis of the chymotrypsin substrate N-succinyl ala-ala-pro-phe ρ -nitroanilide by *L. serricorne* proteinases. These data support the presence of chymotrypsin-like proteinases in *L. serricorne* digestion. Soybean trypsin-chymotrypsin (Bowman Birk) inhibitor is also an inhibitor of trypsin, as well as soybean trypsin (Kunitz) inhibitor. The inhibition of *L. serricorne* proteinases by both soybean inhibitors and the hydrolysis of N- α -benzoyl-L-arginine ρ -nitroanilide suggests that trypsin-like proteinases are also present in the hydrolytic mixture. At low inhibitor concentrations, soybean trypsin (Kunitz) inhibitor and leupeptin caused a slight increase in activity in comparison to control values. Although the cause of these increases is unknown, one explanation is that a proteinase with constrained activity due to proteolysis by another proteinase may be activated indirectly by the inhibitors.

The properties of digestive proteinases in *L. serricorne* have been studied to provide a better understanding for the development of biologically-based pesticides. Results from these studies suggest that protein digestion in *L. serricorne* is primarily due to serine proteinases that are sensitive to selected serine proteinase inhibitors. Digestion of food by serine proteinases is common in lepidopterous insects. Serine proteinases also are important for digestion in some coleopteran species, including species closely related to *L. serricorne*. However, *L. serricorne* can survive on a variety of food sources, and carbohydrases and lipases may also provide nutrition. Targeting these enzymes may also be necessary for the development of effective biopesticides.

Symbiotic yeasts in *L. serricorne* contribute B vitamins when diets are vitamin deficient (Milne, 1963; Pant & Fraenkel, 1950). It is not known whether yeast symbionts influence the proteinase profiles in *L. serricorne*. Yeast symbionts promote *L. serricorne* larval survival when dietary conditions are suboptimal, and compounds toxic to the symbiont, such as sorbic acid, have been suggested as a control agent for *L. serricorne* (Milne, 1963). However, another biological control method could involve the development of transgenic symbionts that express proteinase inhibitors, such as those from soybean, with expression activated by digestion in *L. serricorne*.

Adaptive responses by insects to ingested proteinase inhibitors may complicate inhibitor-based control strategies. Insects can adapt to proteinase inhibitors by compensatory mechanisms (reviewed in Oppert, 2000). These include an increased production of inhibitor-sensitive proteinases, or the synthesis of novel inhibitor-insensitive proteinases. Some coleopteran larvae have demonstrated resistance to proteinase inhibitors by inhibitor proteolysis (Girard *et al.*, 1998). Therefore, candidate serine proteinase inhibitors should be bioassayed for the effect on larval growth and survival. Survivors from inhibitor bioassays will need to be evaluated for changes in proteinase activities prior to the advancement of transgenic technology for effective control of *L. serricorne*.

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