Examination of Midgut Proteinases of the Adult Southern Pine Beetle (Coleoptera: Scolytidae)[1]

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ABSTRACT The midgut of adult southern pine beetles, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae), contains digestive enzymes with optimal proteolytic activity *in vitro* near pH 7. General proteinase activity was significantly inhibited by serine and cysteine proteinase class inhibitors, while limited activation by cysteine proteinase class activators was apparent. These results indicate that both cysteine and serine proteinases are present in the adult midgut. The presence of both proteinase classes in adult southern pine beetles coincides with previous studies showing widespread occurrence of these two classes of proteinases as digestive enzymes in midguts of other coleopteran species, but represents one of few beetle species known to possess both proteinase classes simultaneously.

KEY WORDS Coleoptera, Scolytidae, *Dendroctonus frontalis,* southern pine beetle, midgut, proteinase, proteinase inhibitor.

Proteinases are grouped into four classes: aspartic, serine, cysteine, and metallo-proteinases, and are classified according to characteristics such as range of pH activity, cleavage of specific substrates, and inhibition of activity by specific inhibitors (North 1982). Three of the four proteinase classes have been identified as digestive enzymes in insects (Applebaum 1985). Serine proteinases are the most common, having been identified in Coleoptera (Murdock et al. 1987), Lepidoptera (Broadway 1989, Purcell et al. 1992, Christeller et al. 1992), and Orthoptera (Christeller et al. 1990). Cysteine proteinases have been found in Hemiptera (Houseman and Downe 1982, 1983) and Coleoptera (Murdock et al. 1987), while aspartic proteinases are known from Hemiptera (Houseman and Downe 1980, 1981), Diptera (Pendola and Greenberg 1975), and Coleoptera (Lemos et al. 1990).

Coleopteran digestive proteinases have been widely studied. Serine proteinases have been identified in several families of Coleoptera, including Carabidae (Gooding and Huang 1969, Vaje et al. 1984), Elateridae (Colepicolo-Neto et al. 1987), Chrysomelidae (Wolfson and Murdock 1990), Tenebrionidae (Thie and Houseman 1990), Curculionidae (Purcell et al. 1992), Dermestidae (Baker 1976), and Scarabeidae (Christeller et al. 1989, Christeller and Shaw 1989). Aspartic proteinases have been reported in Meloidae (Wolfson and Murdock 1990), Chrysomelidae (Wolfson and Murdock 1990), Coccinellidae

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(Wolfson and Murdock 1990), and Bruchidae (Lemos et al. 1990). Cysteine proteinases are widespread and common in Coleoptera and have been reported from 10 families (Murdock et al. 1987, Wolfson and Murdock 1987, Wolfson and Murdock 1990, Hines et al. 1990, Thie and Houseman 1990, Purcell et al. 1992).

It is apparent from previous studies that digestive proteinases differ among insect orders and can vary widely even within closer taxonomic groups. As a result, few generalizations can be made from previous studies, and it becomes necessary to examine each species of interest to gain reliable information concerning digestive proteinases. This is further substantiated by the lack of studies on many insect species and families, many of which are of significant economic importance.

We examined the midgut proteinases of adult southern pine beetles, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae). The southern pine beetle is an economically important pest that attacks and kills pine trees (*Pinus* spp.) in the southern United States. In trees that are successfully attacked, adult beetles feed in the phloem-cambium tissues beneath the bark and construct galleries where oviposition occurs (Payne 1980). Examination of digestive proteinases has not been conducted on this beetle or any beetle with a similar feeding habit. The southern pine beetle also represents the family Scolytidae, a group whose digestive proteinases have been unstudied until now.

Materials and Methods

Insects. All insects used were collected from an active southern pine beetle infestation in the Indian Mounds Wilderness Area of the Sabine National Forest in eastern Texas, U.S.A. from May to October 1992. Bark containing southern pine beetle pupae was removed from infested loblolly (*Pinus taeda* L.) and shortleaf (*P. echinata* Miller) pines and returned to the laboratory. Bark was placed in ventilated rearing cans (Cooper and Stephen 1978) and held at 24°C to allow complete development to adults. Adults emerged into collecting jars filled with granular styrofoam, thereby restricting adult feeding and ingestion of host material to the process of emergence from pupal chambers. This step was taken because southern pine beetles are associated with numerous microorganisms (Moore 1972, Bridges et al. 1984), including three symbiotic fungal species (Barras and Perry 1972, Barras 1973), and in many xylophagous insects digestive enzymes can be acquired from associated microorganisms during feeding (Kukor and Martin 1983, 1986a, 1986b, Martin and Martin 1978). Although Moore (1972) found only common bacteria and fungi in a study of southern pine beetle alimentary tracts, it was necessary for this study to minimize the possibility of digestive enzymes being acquired from external sources. Thus, by using newly-emerged adult beetles with minimal feeding time, we feel that the digestive enzymes from the midguts of our study beetles are strictly of beetle origin.

Upon collection insects were sorted to remove dead individuals and live beetles were immediately processed. Two groups of approximately 200 insect midguts were surgically removed and frozen at -60° C overnight (approximately 10 h). On the following day midguts were thawed and placed separately in 1 ml of iso-osmotic saline (0.9% NaCl, 8.5% sucrose). The midguts were homogenized (Tekmar Tissumizer),

then centrifuged (10,000 x g) for 10 minutes at 4°C. The supernatant, which contained both midgut wall and luminal proteinases, was removed and diluted to a final volume of 1 ml with iso-osmotic saline. The following pH and inhibitor/activator studies each used one of these group preparations. Assays were conducted with 40 µl aliquots of the midgut homogenate containing approximately 40 µg of protein. Protein concentration was determined using the methods of Bradford (1976), with bovine serum albumin as the standard.

pH Assays. All proteinase substrates, inhibitors, and activators were purchased from Sigma Chemical Co. Proteinase assays generally follow the methods of Purcell et al. (1992). Aliquots of midgut homogenate were assayed for proteinase activity from pH 3-10 using 1 ml reaction volumes of the general proteinase substrates azocoll present at 0.5% (for pH 3-5) and azocasein present at 1.0% (for pH 6-10). Controls contained an equal aliquot of iso-osmotic saline in place of midgut homogenate. Azocasein assays were conducted in replicate at 30° C for 2 h. At the end of incubation undigested azocasein was precipitated by the addition of 1 ml of cold 10% trichloroacetic acid (TCA). The TCA precipitate was pelleted by centrifugation for 10 min. The supernatant was removed and diluted to 50% by adding an equal quantity of 0.1 M NaOH and the absorbance read at 450 nm on a Milton-Roy Spectronic 3000 Array spectrophotometer. Azocoll assays were conducted in replicate at 30°C for 3 h with constant stirring to maintain uniform suspension of the substrate. Undigested azocoll at the end of incubation was pelleted by centrifugation for 10 min. The supernatant was removed and the absorbance read at 525 nm. The following buffers were used: pH 3-5, 0.1 M citric acid/0.2 M dibasic sodium phosphate; pH 6-8, 0.1 M potassium phosphate; pH 9-10, 0.2 M glycine/0.2 M NaOH. For the pH assay, average activities are expressed as a percentage of the activity at the optimum pH, which is assigned a value of 100%.

Inhibitor/Activator Assays. By using specific inhibitors and activators for each of the four proteinase classes, it is possible to classify the unknown proteinases in the midgut homogenate (Dingle and Gordon 1986). Proteinase inhibitor assays were conducted in replicate at the optimum pH of proteinase activity (Purcell et al. 1992). The inhibitors and activators used and their concentrations were as follows: EDTA (a metallo-proteinase inhibitor), 1 mM; soybean trypsin inhibitor (STI) (a serine proteinase inhibitor [Type I-S, highest purity available]), 10 µg/ml; Bowman-Birk inhibitor (BBI) (a serine proteinase inhibitor), 10 µg/ml; E-64 (a cysteine proteinase inhibitor), 50 µM; pepstatin (an aspartic proteinase inhibitor), 20 µM; cysteine (a cysteine proteinase activator), 5 mM; and cysteine + EDTA (a cysteine proteinase activator treatment), 5 mM and 1 mM, respectively. One ml volumes of azocasein present at 1.0% were used as the substrate. Equal volumes of midgut fluid and solutions containing either an inhibitor or activator at its respective concentration were preincubated for 20 min at 4°C before adding to the substrate. Azocasein assays were conducted as above. Average activities are expressed as a percentage of a control treatment, which had no inhibitors or activators present and was assigned a value of 100%.

Results

Highest midgut homogenate proteinase activity occurred in the neutral to slightly alkaline range (pH 7-8). Midgut homogenate activity was completely inactive at pH 10, and was relatively inactive below a pH of 6 (Fig. 1).

Proteinase inhibitors and activators were tested against southern pine beetle adult midgut homogenates at pH 7, where maximum proteinase activity was found. Midgut homogenate proteinase activity showed slight but insignificant inhibition by EDTA (Fig. 2), indicating that metallo-proteinases might be only a minor component of adult midgut proteinases. Proteinase activity was significantly reduced by serine and cysteine inhibitors. The serine inhibitors STI and BBI reduced proteinase activity by 87% and 83%, respectively (Fig. 2), indicating the presence of trypsin-like serine proteinases in adult midgut homogenates. E-64 inhibited proteinase activity of adult midgut homogenates by 76%, indicating the presence of cysteine proteinases. Pepstatin, an aspartic proteinase inhibitor, had no significant effect on proteinase activity. The presence of cysteine and cysteine + EDTA showed increased proteinase activity to 134%

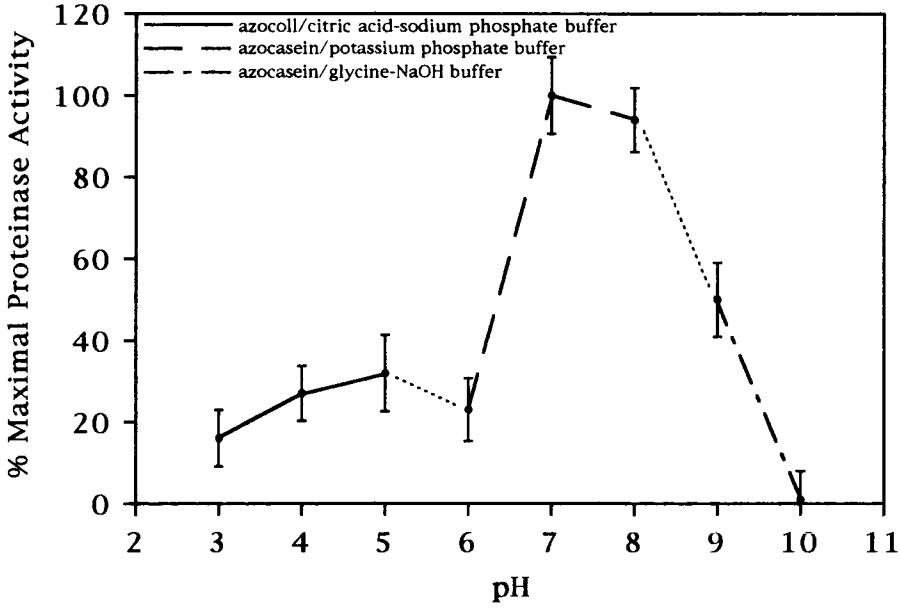

Fig. 1. pH optima curve for proteinase activity of adult southern pine beetle midgut homogenate. Aliquots of midgut homogenate were assayed using the proteinase substrates azocoll and azocasein. See text for explanation of buffer system. Average (with 95% confidence interval) activity is expressed as a percentage of the optimal pH (7), which is assigned a value of 100%.

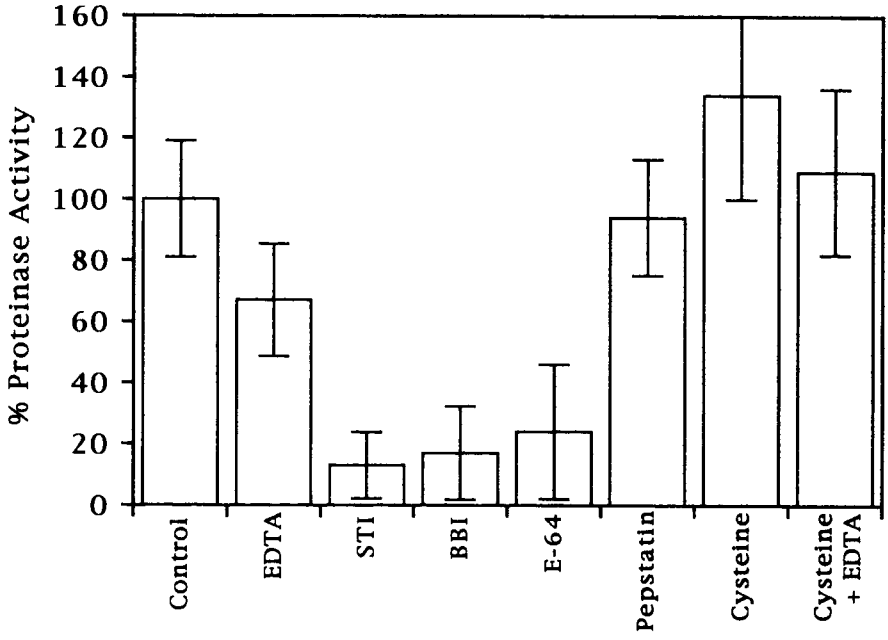

Fig. 2. Effects of various proteinase inhibitors or activators on proteinase activity of midgut homogenate of adult southern pine beetles. The assays were conducted at pH 7 using azocasein as a substrate. Average (with 95% confidence interval) activity is expressed as a percentage of the activity in the control treatment, which had no inhibitors or activators present and was assigned a value of 100%.

and 110% of the control level, respectively, a further indication of cysteine proteinases in the midgut homogenates, although statistically the levels of activation were insignificant. The lower level of activation in the cysteine + EDTA treatment in comparison with the cysteine treatment may again be an indication of the presence of small amounts of metallo-proteinases in the midgut homogenates being inhibited by EDTA. Significant inhibition by the STI, BBI, and E-64 treatments confirm that serine and cysteine proteinases, however, are the major components of the midgut proteinases of the southern pine beetle.

Discussion

Other coleopteran species reported to have digestive proteinases with high proteolytic activity near pH 7 include the southern corn rootworm (Purcell et al. 1992), the Colorado potato beetle (Purcell et al. 1992), the grass grub larvae, *Costelytra zealandica* (Christeller et al. 1989), a species of ground beetle, *Pterostichus melanarius* (Gooding and Huang 1969), and a bruchid beetle,

Carydes brasiliensis (Wolfson and Murdock 1990). Of these beetles, 2 possessed serine proteinases (*C. zealandica* and *P. melanarius*) and 2 possessed cysteine proteinases (southern corn rootworm and Colorado potato beetle). All of these beetles vary in diet and include both phytophagous and predaceous species, confirming that diet alone is not a reliable indicator of which class of digestive proteinases a given species might possess.

Cysteine proteinases are widespread in Coleoptera (Murdock et al. 1987, Wolfson and Murdock 1990), as are serine proteinases (Purcell et al. 1992, Christeller et al. 1989, Baker 1976, Gooding and Huang 1969). However, only a few beetle species have been previously reported to possess both. Thie and Houseman (1990) found both cysteine and serine proteinases in the larvae of the yellow mealworm (*Tenebrio molitor*). Wolfson and Murdock (1990), in a survey of digestive proteinases of a variety of insect species, reported 5 species of beetles having more than one class of digestive proteinase. The southern pine beetle appears to fit in a small group of coleopterans which possess more than one class of proteinase. Thie and Houseman (1990) suggested that coleopteran species could be divided into 3 groups based on the presence of either serine or cysteine proteinases, or a combination of both. Our results support a classification of this kind, although more investigations are needed to determine the extent of multiple digestive proteinase classes in coleopterans.

Studies of insect proteinases are usually conducted on phytophagous species in relation to their diet (Applebaum 1985) of various plant tissues. Naturally-occurring proteinase inhibitors in plants are thought to be a defense mechanism against herbivory (Green and Ryan 1972, Ryan and Green 1974) and have been suggested as a possible method of insect control (Broadway and Duffey 1986, Murdock et al. 1988, Shukle and Murdock 1983, Wolfson and Murdock 1987). This mechanism has led to the ongoing production of transgenic plants with gene expression for proteinase inhibitors (Hilder et al. 1987, Johnson et al. 1990). While transgenic pines with gene expression for proteinase inhibitors are not available for the control of the southern pine beetle, a defense system of pines may possess a similar inhibitory function. Pines respond to bark beetle attack by producing a resin-soaked necrotic lesion (Berryman 1972) which forms in the phloem-cambium tissue layers at the point of beetle attack (Raffa and Berryman 1983). Larval southern pine beetles forced to feed in this lesion tissue have a higher rate of mortality and slower development to emergence than larvae in normal tissue (Paine and Stephen 1988). The presence of inhibitors in the diets of other insect larvae also have been shown to increase mortality and retard development (Broadway and Duffey 1986, Wolfson and Murdock 1987, Murdock et al. 1988, Hines et al. 1990), although the presence of inhibitors in pine tissue has never been investigated. However, because the wounding of plant tissue by insect feeding may cause an increase in plant proteinase inhibitors (Green and Ryan 1972, Ryan and Green 1974), it is not unreasonable to suggest that pines may also possess proteinase inhibitors as a defense mechanism against insect herbivory.

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