

## Characteristics of digestive proteases in the gut of some insect orders

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### Abstract

Gut proteases of 20 species from 13 insect orders were characterized by activity staining after polyacrylamide gel electrophoresis and the effect of class-specific protease inhibitors on their zymogram. Species in Orthoptera, Dictyoptera, Lepidoptera and Hymenoptera mainly had serine proteases in their gut. Those in Ephemeroptera, Odonata, Plecoptera and Hemiptera had cysteine proteases. Those in Coleoptera, Neuroptera, Mecoptera and Diptera had both serine+cysteine proteases in their gut. The protease class of each species tended to reflect phylogenetic relationship rather than feeding habits.

**Key words:** Digestive proteases, protease inhibitors, insect orders

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### INTRODUCTION

Digestive proteases have been characterized in a variety of insects and especially well in Orthoptera, Dictyoptera, Hemiptera, Coleoptera, Lepidoptera, Diptera and Hymenoptera because of their agricultural and medical importance. Terra et al. (1996a) reviewed the characteristics of many insects' digestive proteases. In order to examine whether the nature of digestive proteases is attributed to feeding habits or evolutionary relationship, I studied the protease class in the guts of 20 species from 13 orders including the following orders which represent the first such reports; Ephemeroptera, Odonata, Plecoptera, Trichoptera, Neuroptera and Mecoptera.

Recently, the genetic engineering plants introducing protease inhibitor genes have been proposed to be able to control insect pests (Hilder et al., 1987; Johnson et al., 1989). The classification of digestive proteases in the gut of agricultural insect pests is necessary for developing transgenic plants resistant to the pests.

In this study, the digestive proteases in the gut of 20 species from 13 insect orders were characterized by activity staining after polyacrylamide gel electrophoresis and the effect of class-specific protease inhibitors on their zymogram.

### MATERIALS AND METHODS

**Insects.** Insects used for assay are shown in Table 1. Twenty species from 13 orders were tested. Larvae of *Riptortus clavatus*, which were reared on soy bean seeds were received from Mr. A. Kikuchi (National Agriculture Research Center), those of *Eocanthecona furcellata*, which were reared on larvae of *Spodoptera litura* were received from Dr. S. Wakamura (National Institute of Sericultural and Entomological Science), those of *Chrysopa carnea*, which were reared on eggs of *Tribolium castaneum* were received from Mr. T. Kubota (National Institute of Agro-Environmental Sciences), those of *Chilo suppressalis* were reared on rice seedlings and those of *Delia antiqua* were reared on onion bulbs. Larvae of *Periplaneta americana*, which were reared on a diet for guinea pigs (GC-4<sup>®</sup>, Oriental Kobo Co.), and those of *Psacotea hilaris*, which were reared on an artificial diet for the silkworm, *Bombyx mori* (Yakuruto Co.), were received from Drs. Y. Shintani and Y. Ishikawa (The University of Tokyo). Other species were collected in the field.

**Abbreviations.** DAN, diazoacetylnorleucin methyl ester; DMSO, dimethyl sulfoxide; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane; EDTA, ethylenediamine tetra-acetic acid; PAGE, polyacrylamide gel electrophoresis; pHMB, 4-(hydroxymercurio)benzoate; PMSF,

Table 1. Insects examined in the current study

Species	Stage	Feeding habit	Food
Ephemeroptera			
Ephemerellidae			
<i>Ephemerella trispina</i>	larva	herbivore	algae etc.
Odonata			
Aeschnidae			
<i>Aeschna juncea</i>	adult	carnivore	small insects
Plecoptera			
Perlidae			
<i>Acroneuria stigmatica</i>	larva	detritivore	detritus, algae, etc.
Orthoptera			
Locustidae			
<i>Oxya yezoensis</i>	adult	herbivore	rice leaves
Dictyoptera			
Mantidae			
<i>Statilia maculata</i>	adult	carnivore	insects
Blattidae			
<i>Periplaneta americana</i>	larva	omnivore	various things
Hemiptera			
Coreidae			
<i>Riptortus clavatus</i>	adult	herbivore	soybean seeds etc.
Pentatomidae			
<i>Eocanthecona furcellata</i>	adult	carnivore	insect blood
Aphididae			
<i>Prociphilus</i> sp.	larva	herbivore	plant phloem sap
Coleoptera			
Cerambycidae			
<i>Psciothea hilaris</i>	larva	herbivore	mulberry woods
Scarabaeidae			
<i>Blitopertha</i> sp.	larva	scavenger	leaf mold
Curculionidae			
<i>Lissorhoptrus oryzophilus</i>	larva	herbivore	rice roots
Carabidae			
<i>Carabus</i> sp.	larva	carnivore	insects
Neuroptera			
Chrysopidae			
<i>Chrysopa carnea</i>	larva	carnivore	insect blood
Mecoptera			
Panorpidae			
<i>Panorpa</i> sp.	adult	carnivore	insects
Diptera			
Anthomyiidae			
<i>Delia antiqua</i>	larva	herbivore	onion bulbs
Trichoptera			
Stenopsychidae			
<i>Stenopsyche griseipennis</i>	larva	detritivore	detritus, algae, etc.
Lepidoptera			
Pyrilidae			
<i>Chilo suppressalis</i>	larva	herbivore	rice leaves
Hymenoptera			
Tenthredinidae			
<i>Dolerus japonicus</i>	larva	herbivore	leaves of <i>Equisetum</i> sp.
Vespididae			
<i>Polistes chinensis</i>	larva	carnivore	insects

phenylmethanesulfonyl fluoride; SDS, sodium dodecylsulfate; TLCK, 1-chloro-3-tosylamido-7-amino-L-2-heptanone; TPCK, 1-chloro-3-tosylamido-4-phenyl-L-2-butanone.

#### Preparation of digestive fluid from insect gut.

Digestive tracts including the gut contents were carefully dissected from the insect body in the ice cold phosphate-buffered saline (PBS, pH 7.2) containing 1 mM PMSF under a microscope. One or more intact digestive tracts were collected in a 1.5 ml Eppendorf tube chilled on ice and centrifuged for 5 min at 15,000 rpm at 4°C. The resulting supernatant (luminal contents) was collected and stored at -20°C until use.

**Gel electrophoresis.** Electrophoretic mobility of proteolytic activity on gelatin containing 10% SDS-PAGE gels was determined by the method of Gillikin et al. (1992) with the following modification: 0.25% gelatin was replaced with 0.2% gelatin. In a preliminary experiment, the luminal contents were sufficient to detect proteolytic activity at a concentration of 1 : 1,000. Applying the concentrated fluid, a smear zymogram pattern was observed. Thus, each luminal contents was diluted to 8 ml per tract, except for *Prociophilus* sp. whose luminal contents was used without dilution. Eight microliters of the diluted luminal contents was mixed with 4 µl of SDS-PAGE sample buffer (2% SDS, 20% glycerol, 200 mM Tris-HCl (pH 7.0) and 0.1% bromophenol blue). The samples were loaded onto a stacking gel and electrophoresed at 8 mA at 4°C. After electrophoresis, the gel was washed in 2.5% Triton X-100 for 30 min to remove the SDS within the gel, rinsed twice with distilled water and transferred to 50 mM Tris-HCl (pH 8.0), 10 mM CaCl<sub>2</sub>, for 10 min to remove the Triton X-100. Then each gel lane was cut into strips and separately incubated for 16 h at 37°C with the same buffer as above containing various inhibitors. The inhibitors used here were as follows: 5 mM EDTA, 1 mM *o*-phenanthroline; 3 mM PMSF, 1 mM TPCK, 0.7 mM TLCK; 10 µM leupeptin, 1 mM pHMB, 0.1 mM E-64; and 5 mM DAN. After incubation, the strips of gel were stained for 2 h with 0.25% Coomassie® brilliant blue G-250 containing 50% methanol and 10% acetic acid. Proteolytic activity was visualized as transpar-

ent bands on a blue background after destaining in 25% methanol and 7% acetic acid for 6 h.

**Chemicals.** The inhibitors were stock solutions made as follows and diluted to the appropriate concentrations in the incubation buffer just before use. Five hundred millimolar EDTA, *o*-phenanthroline and 10 mM leupeptin were dissolved in water to make stock solutions. One hundred millimolar TPCK and 70 mM TLCK were dissolved in DMSO; 1 M pHMB was in 0.1 M NaOH; 10 mM E-64 was in 70% methanol; 5 M DAN was in 100% methanol; and 100 mM PMSF was dissolved in 2-propanol and stored at -20°C.

## RESULTS AND DISCUSSION

Examples of the effect of class-specific protease inhibitors on the zymogram of the gut proteases are shown in Fig. 1. Proteolytic activity was visualized as transparent bands in a black background, because of the digestion of gelatin as a substrate in the gel. The proteolytic nature of each enzyme (each band) on the gel was determined by comparing between those of the control (without inhibitors) and those with each class-specific inhibitor. When a proteolytic activity of an enzyme was inhibited by an inhibitor, the band corresponding to the control lane was stained and invisible. The results are summarized in Table 2. EDTA and *o*-phenanthroline are typical metalloprotease inhibitors. PMSF, TPCK and TLCK are serine protease inhibitors. TPCK is a chymotrypsin inhibitor and TLCK is a trypsin inhibitor, however, they also inhibit a histidine active site of cysteine proteases. Leupeptin is an inhibitor of both serine and cysteine proteases. pHMB and E-64 are typical cysteine protease inhibitors. DAN is an aspartic acid protease inhibitor.

The gut proteases of *Ephemerella trispina* (Ephemeroptera; Ephemerellidae) were inhibited by leupeptin, pHMB and E-64, showing the existence of cysteine proteases.

The gut proteases of *Aeschna juncea* (Odonata; Aeschnidae) were inhibited by TLCK, leupeptin and E-64. E-64 sensitive activity suggested that they were cysteine proteases. The pHMB insensitive nature is unclear. The TLCK sensitive nature may have resulted

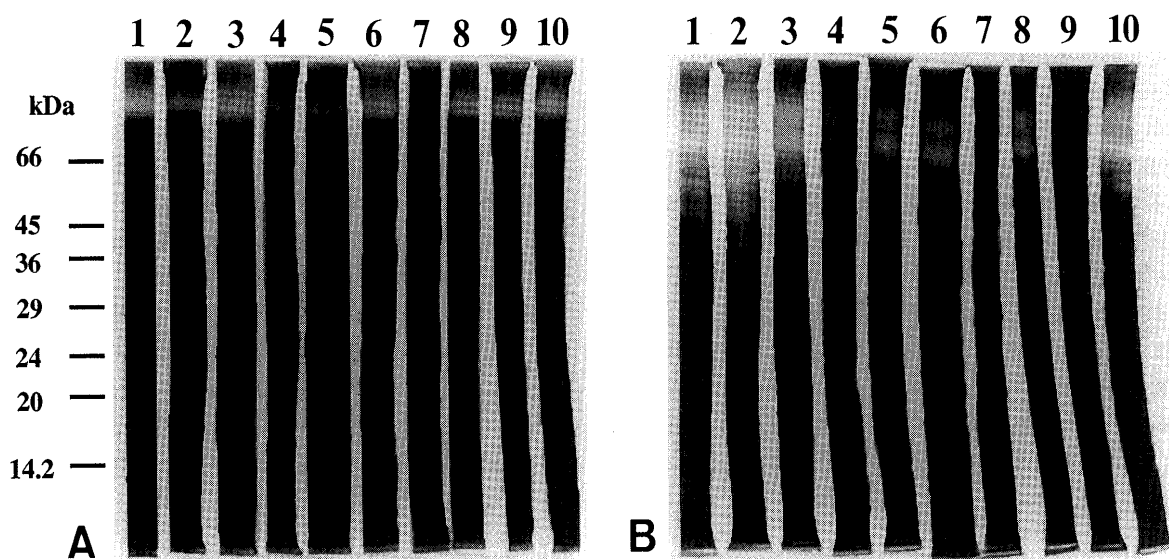


Fig. 1. Effects of inhibitors on the zymogram of insect gut proteases, A: *Oxya yaezoensis* and B: *Panorpa* sp. The luminal contents were subjected to electrophoresis in a gelatin-containing gel and the lanes were sectioned and incubated separately with the following inhibitors. 1, control (without inhibitors); 2, EDTA; 3, *o*-phenanthroline; 4, PMSF; 5, TPCK; 6, TLCK; 7, leupeptin; 8, pHMB; 9, E-64; 10, DAN.

from a histidine active site of cysteine proteases (Rawlings and Barrett, 1993).

The gut proteases of *Acroneuria stigmatica* (Plecoptera; Perlidae) were inhibited by TLCK, leupeptin and E-64, suggesting cysteine proteases. The pHMB insensitive and TLCK sensitive natures were similar to those of *A. juncea*.

The gut proteases of *Oxya yaezoensis* (Orthoptera; Locustidae) were inhibited by EDTA, PMSF, TPCK and leupeptin but neither by pHMB nor by E-64. It is suggested that *O. yaezoensis* has EDTA sensitive metalloproteases and serine (chymotrypsin-like) proteases.

The gut proteases of two species of Dictyoptera, *Statilia maculata* (Mantidae) and *Periplaneta americana* (Blattidae) were inhibited by PMSF, TLCK and leupeptin, suggesting that they are serine proteases, especially trypsin. The gut proteases of *P. americana* were also inhibited by EDTA, indicating the existence of metalloproteases.

In Hemiptera, the gut proteases of *Riptortus clavatus*, a soybean seed feeder, were inhibited by *o*-phenanthroline, TPCK, leupeptin and E-64. Those of *Eocanthecona furcellata*, insect blood sucking species, were inhibited by TLCK, leupeptin, pHMB and E-64. The two species have cysteine proteases in their guts, in spite of their different feeding habits. TPCK and TLCK

may inhibit a histidine active site of cysteine proteases. *o*-Phenanthroline-sensitive metalloproteases were found in the gut of *R. clavatus*. No proteolytic activities were detected in the gut of the phloem sucking aphid, *Prociphilus* sp. (data not shown). Terra and Ferreira (1994) reviewed that the blood sucking and seed sucking heteropteran insects have cysteine proteases in their guts but that the phloem and xylem sucking homopteran insects did not have proteases in their guts.

For Coleoptera, I examined the proteases of the following species; *Psciothea hilaris* (Cerambycidae), *Blitopertha* sp. (Scarabaeidae), *Lissorhoptrus oryzophilus* (Curculionidae) and *Carabus* sp. (Carabidae). All of the proteases in their guts had an E-64 inhibited nature, suggesting the existence of cysteine proteases. The proteases in the guts of *P. hilaris*, *Blitopertha* sp. and *L. oryzophilus* were also inhibited by pHMB. The gut proteases of *P. hilaris* were inhibited by PMSF and TPCK. This suggests the existence of serine proteases in the gut, however it can not be determined whether the TPCK sensitive nature results from a chymotrypsin or a histidine active site of cysteine proteases. *o*-Phenanthroline-sensitive metalloproteases were detected in the gut proteases of *Blitopertha* sp. and *Carabus* sp. Terra and Ferreira (1994)

Table 2. Interaction between gut proteases and class specific inhibitors

Species	5 mM EDTA	1 mM o-Phe	3 mM PMSF	1 mM TPCK	0.7 mM TLCK	10 $\mu$ M Leu	1 mM pHMB	0.1 mM E-64	5 mM DAN	Protease class
Ephemeroptera										
<i>E. trispina</i>						++	+	+		cysteine
Odonata										
<i>A. juncea</i>					+	++		+		cysteine
Plecoptera										
<i>A. stigmatica</i>					+	++		+		cysteine
Orthoptera										
<i>O. yezoensis</i>	+		+	+		++				metallo + serine (chymotrypsin)
Dictyoptera										
<i>S. maculata</i>			+		+	++				serine (trypsin)
<i>P. americana</i>	+		+		+	++				metallo + serine (trypsin)
Hemiptera										
<i>R. clavatus</i>		+		+		+		+		metallo + cysteine
<i>E. furcellata</i>					+	+	+	+		cysteine
Coleoptera										
<i>P. hiliaris</i>			+	+		++	+	+		serine + cysteine
<i>Blitopertha</i> sp.		+			++	++	+	+		metallo + cysteine
<i>L. oryzophilus</i>		+				++	+	++		metallo + cysteine
<i>Carabus</i> sp.				+	+	++		+		cysteine
Neuroptera										
<i>C. carnea</i>		+	+	+	+	++	+	++		metallo + serine + cysteine
Mecoptera										
<i>Panorpa</i> sp.		+	+	+	+	++	+	+		metallo + serine + cysteine
Diptera										
<i>D. antiqua</i>		+	+	+	++	++		++		metallo + serine + cysteine
Trichoptera										
<i>S. griseipennis</i>					++	++				?
Lepidoptera										
<i>C. suppressalis</i>		+	+		++	++				metallo + serine (trypsin)
Hymenoptera										
<i>D. japonicus</i>			+		++	++				serine (trypsin)
<i>P. chinensis</i>		+	+		+	+				metallo + serine (trypsin)

+, partially inhibited; ++, strongly inhibited.

o-Phe, *o*-phenanthroline; Leu, leupeptin.

summarized that cysteine proteases were found in the gut of insects belonging to Cucujiformia but not from the following families: Cerambycidae, Dermestidae, Scarabaeidae, Elateridae and Carabidae. However, I found cysteine proteases in the gut of species in the following families: Cerambycidae, Scarabaeidae and Carabidae. The ancestor of not only Cucujiformia but also the other suborders in Coleoptera might use serine proteases and cysteine proteases derived from lysosomes, and most of them might lose serine proteases because of the rich concentration of trypsin inhibitors in their food

(Terra and Ferreira, 1994) or some other unknown reasons during their evolution. Some species, such as *Tenebrio molitor* (Thie and Houseman, 1990) and *P. hiliaris*, might not have lost trypsin-like enzymes.

The gut proteases of *Chrysopa carnea* (Neuroptera; Chrysopidae) were inhibited by *o*-phenanthroline, PMSF, TPCK, TLCK, leupeptin, pHMB and E-64. These data suggest that *C. carnea* will have metallo-, serine and cysteine proteases.

The gut proteases of *Panorpa* sp. (Mecoptera; Panorpidae) were also inhibited by *o*-

phenanthroline, PMSF, TPCK, TLCK, leupeptin, pHMB and E-64 suggesting the existence of metallo-, serine and cysteine proteases.

The gut proteases of a phytophagous onion maggot, *Delia antiqua* (Diptera; Anthomyiidae), were inhibited by *o*-phenanthroline, PMSF, TPCK, TLCK, leupeptin, pHMB and E-64 suggesting the existence of metallo-, serine and cysteine proteases.

The gut proteases of *Stenopsyche griseipennis* (Trichoptera; Stenopsychidae) were inhibited only by TLCK and leupeptin. The proteases cannot be classified from these data. More detailed study will be needed.

The gut protease of *Chilo suppressalis* (Lepidoptera; Pyralidae) was reported to be trypsin (Yushima and Ishii, 1952). In this study, it was confirmed by inhibition of PMSF and TLCK. The gut protease of *C. suppressalis* was also inhibited by *o*-phenanthroline, suggesting the existence of metalloproteases.

The gut proteases of the two hymenopteran insects, a phytophagous species, *Dolerus japonicus*, and a carnivorous species, *Polistes chinensis* were inhibited by PMSF, TLCK and leupeptin, suggesting serine proteases (trypsin). Metalloproteases were also found in the gut of *P. chinensis*.

According to the review of Terra et al. (1996a), the metal ion independent enzymes, metalloproteases, aminopeptidases and metallo-carboxypeptidases have been reported from the gut proteases in species of Orthoptera, Hemiptera, Coleoptera, Diptera, Lepidoptera and Hymenoptera. Serine proteases have been found in the gut proteases of species of Orthoptera, Dictyoptera, Coleoptera, Diptera, Lepidoptera and Hymenoptera, and cysteine proteases have been found in those of the species of Hemiptera and Coleoptera. In this study, the following new data could be added: a species of Ephemeroptera, Odonata and Plecoptera have cysteine proteases, a species of Neuroptera and Mecoptera have metallo-, serine and cysteine proteases, *D. antiqua*, a dipteran species, has cysteine proteases as well as metallo- and serine proteases in their guts. Aspartic acid proteases have been reported in the gut of species in Hemiptera, Coleoptera and Diptera (Terra et al., 1996a). In the current study,

aspartic acid proteases were not detected in the gut proteases of any species examined. Since the optimum pH was around 3.5 (Houseman and Downe, 1983; Lemos and Terra, 1991; Silva and Xavier-Filho, 1991), the activity might not be detected at the pH 8.0 condition used in this study.

Terra et al. (1996b) proposed the view that the types of digestive proteases in the gut seem to be similar within the same taxon with different feeding habits. In contrast, insects with similar feeding habits, but within different taxa have a different protease nature. Some data which support this theory were recognized in this study. For example, the gut proteases of two heteropteran species of *R. clavatus*, soybean seed feeder, and *E. furcellata*, insect blood sucking species were the same cysteine proteases. The gut proteases of two hymenopteran insects, a phytophagous species, *D. japonicus*, and a carnivorous species, *P. chinensis*, were the same serine proteases. The feeding habits of the following three species are different; the orthopteran species of *O. yezoensis* is a plant feeder, and the dictyopteran species of *S. maculata* is an animal feeder and *P. americana* is a generalist, however the main proteases in their guts are the same serine proteases. These species belong to the same monophyletic taxon, Orthopteroidea, and are supposed to have evolved from the same ancestor (Kristensen, 1981). Similar instances were seen in the gut proteases of both ephemeropteran species and odonatan species which were cysteine proteases, despite their different feeding habits, and this coincides with the monophyly hypothesis of Ephemeroptera+Odonata (Hennig, 1981). In contrast, *O. yezoensis* (Orthoptera), *L. oryzophilus* (Coleoptera) and *C. suppressalis* (Lepidoptera) have similar feeding habits, their host plants are mainly rice plants, but the class of their gut proteases are different. Serine proteases of the guts of Orthoptera and Lepidoptera may be attributed to an evolutionary convergence.

The digestive protease class in the gut of agricultural pests, for example, *O. yezoensis*, *L. oryzophilus* and *C. suppressalis*, will provide useful information for developing transgenic plants resistant to pests.

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