



cDNAs for a Chymotrypsinogen-like Protein from Two Strains of *Plodia interpunctella*

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Gut proteinases are involved in the solubilization and activation of insecticidal toxins produced by *Bacillus thuringiensis* and may also be involved in resistance development. Approximately threefold lower chymotrypsin-like enzyme activity was observed in a Bt(*entomocidus*)-resistant strain of the Indianmeal moth, *Plodia interpunctella*, than that in the Bt-susceptible strain. Because chymotrypsin-like proteinases are involved in Bt protoxin activation in *P. interpunctella*, we compared cDNA sequences, mRNA expression levels, and genomic DNA for chymotrypsin-like enzymes in Bt-susceptible and Bt-resistant strains of *P. interpunctella*. To isolate cDNA coding for chymotrypsinogen-like proteinases, a probe was developed using polymerase chain reaction (PCR) amplification of a cDNA library from the Bt-susceptible strain using a vector primer and a degenerate primer corresponding to a conserved sequence in the active site of serine proteinases. This probe was used to screen cDNA libraries from resistant and susceptible strains. Predicted amino acid sequences from cDNA clones of each strain share similarity with sequences of chymotrypsin-like proteinases and are most similar to a chymotrypsin-like proteinase from the tobacco hornworm, *Manduca sexta*. cDNAs for putative chymotrypsinogen-like proteins from both Bt-susceptible and Bt-resistant strains of *P. interpunctella* share an identical open reading frame of 846 nucleotides. The encoded proteins contain amino acid sequence motifs of serine proteinase active sites, disulfide-bridge cysteine residues, and both zymogen activation and signal peptides. A difference between these cDNAs was observed only in the untranslated region where a substitution of guanine for adenine occurred in the Bt-resistant strain. Southern and Northern blotting analyses indicated that there are no major differences in chymotrypsinogen-like genomic organization and mRNA expression in the two strains. These data suggest that chymotrypsinogen-like proteinase genes and their transcription are similar in the Bt-susceptible and Bt-resistant strains of *P. interpunctella*. Published by Elsevier Science Ltd

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INTRODUCTION

Bacillus thuringiensis δ -endotoxins (Bt) are an alternative to traditional chemical agents for managing many economically important insects. Unfortunately, the control of insect pests with environmentally safe Bt toxins is threatened due to the development of resistant strains (McGaughey and Whalon, 1992; McGaughey and Oppert, 1996). For example, the Indianmeal moth, *Plodia interpunctella* (Hübner), is capable of adapting to Bt and

resistant strains can rapidly develop which are several orders of magnitude more tolerant of Bt than the parent strain (McGaughey, 1985a, b).

Many Bt toxins are lethal to lepidopteran insects that typically possess midguts with an alkaline pH and serine proteinases as major digestive enzymes. Proteinases are involved in solubilizing and activating Bt protoxins, which allows for the binding of toxins to target tissues. Because gut proteinases play a certain role in Bt toxicity in susceptible insects, researchers have proposed a proteinase-mediated resistance mechanism. Recent evidence indicates that altered gut proteinases may enable some insects to adapt to Bt toxins. Oppert *et al.* (1994, 1996, 1997b) reported that an *entomocidus*-resistant strain of *P. interpunctella* had low gut proteinase activity and was unable to activate Bt protoxin as efficiently as Bt-suscep-

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tible and *kurstaki*-resistant strains. In the tobacco budworm, *Heliothis virescens* (Fab.), not only was a Bt-resistant strain unable to activate the protoxin fully, but degradation of the active toxin was accelerated by gut proteinases (Forcada *et al.*, 1996).

Lepidopteran insects have multiple serine proteinases with a variety of specificities, such as trypsin-like, chymotrypsin-like, and elastase-like (Ahmad *et al.*, 1980; Houseman *et al.*, 1989; Christeller *et al.*, 1992; Houseman and Chin, 1995; Johnston *et al.*, 1995; Rymerson and Bodnaryk, 1995; Oppert *et al.*, 1996). Several insect proteinase cDNAs have been cloned and sequenced, including trypsin-like and chymotrypsin-like enzyme cDNAs from the spruce budworm, *Choristoneura fumiferana* (Clemens) (Wang *et al.*, 1993, 1995), tobacco hornworm, *Manduca sexta* (L.) (Peterson *et al.*, 1994, 1995), and the yellow fever mosquito, *Aedes aegypti* (L.) (Barillas-Mury *et al.*, 1991; Noriega *et al.*, 1996). Information about the genes for these insect gut proteinases is important to help understand how they relate to dietary protein digestion, Bt protoxin activation, and resistance development.

The major digestive enzymes of *P. interpunctella* appear to be similar to chymotrypsin and trypsin in their specificity (Johnson *et al.*, 1990; Christeller *et al.*, 1992; Oppert *et al.*, 1994, 1996). Because proteolytic enzymes may be involved in resistance to Bt in *P. interpunctella* (Oppert *et al.*, 1994, 1996, 1997b) and chymotrypsin-like enzymes activate Bt protoxins (Dai and Gill, 1993), we investigated chymotrypsin-like enzyme cDNAs, genomic DNA, and mRNAs from Bt-susceptible and Bt-resistant strains of *P. interpunctella*. The sequences of the cDNAs and encoded chymotrypsinogen-like proteins from two strains of *P. interpunctella* are reported here.

MATERIALS AND METHODS

Insect cultures

P. interpunctella strain RC688^s (Bt-susceptible strain) was collected from farm-stored grain in Riley County, Kansas, USA, and maintained on a diet described by McGaughey and Beeman (1988). A Bt-resistant strain, HD198^r, was selected from RC688^s using Bt subsp. *entomocidus* (McGaughey and Johnson, 1992).

Microplate and proteinase activity blot assay

Late fourth instar larvae were briefly chilled, and the posterior and anterior ends were removed. Guts were excised, immediately submersed in ice-cold 200 mM Tris (pH 8.0) 20 mM CaCl₂ (buffer A), aliquoted 1 per 25 μ l of buffer, and frozen at -20°C for up to 2 weeks until assayed.

Microplate assays were performed using a modified procedure of Oppert *et al.* (1997a). Frozen guts in buffer were thawed, spun at 15,000 g for 5 min, the supernatant was diluted 1:100 in buffer A, and 50 μ l were added to a microplate well. A 100 mg/ml stock solution of N-

succinyl-ala-ala-pro-phe ρ -nitroanilide (SAAPFpNA, Sigma Chemical Co., St Louis, MO, U.S.A.) in dimethyl formamide was diluted 1:100 in buffer A, and 50 μ l were added to each well to initiate the reaction (final SAAPFpNA concentration was 0.8 mM). After a 30 s incubation at 37°C, absorbance was monitored at 405 nm at 15 s intervals over a 5 min period, and the change in absorbance per min per mg of protein in each gut extract was calculated by the software KinetiCalc3 (Biotek, Winooski, VT). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard and the Coomassie® Plus Microtiter Plate Assay (Pierce Chemical Co., Rockford, IL).

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), frozen guts were thawed and spun at 15,000 g for 5 min. Fifteen microliters of supernatant (approximately 0.6 gut equivalents) were combined 1:1 with SDS sample buffer and subjected to SDS-PAGE using 10–20% tricine gels. Proteins were transferred from the gels to nitrocellulose (BA-S; Schleicher and Schuell) and activity blots were processed as previously described (Oppert *et al.*, 1996) using a modified procedure of Ohlsson *et al.* (1986). Briefly, blotted proteins were incubated in 5 ml of buffer A containing 0.5 mg/ml SAAPFpNA for 30 min at 37°C with gentle agitation, using Econoblot sheets (Lablogix Inc. Belmont, CA, U.S.A.) with small volumes. Diazotized nitroanilide was produced by 5 min successive incubations in 0.1% sodium nitrite in 1 M HCl, 0.5% ammonium sulfamate in 1 M HCl, and finally 0.05% N-(1-naphthyl)-ethylenediamine in 47.5% ethanol until a red color appeared. Membranes were placed in heat-sealed bags and stored at -20°C. MultiMark molecular mass standard proteins were from Novex.

RNA and poly(A) RNA purification

Two hundred and fifty fourth instar larvae (approximately 2.5 g wet weight) from each strain were ground in liquid nitrogen. Total RNA was extracted with guanidine thiocyanate denaturing solution and precipitated with isopropanol (Titus, 1991). The poly(A) RNA was isolated from the total RNA by chromatography on an oligo(dT)-cellulose column (Gibco BRL Life-Technologies, Gaithersburg, MD, U.S.A.).

Construction of cDNA libraries

Double-stranded cDNA was synthesized using 5 μ g of poly(A) RNA as a template (ZAP-cDNA synthesis kit; Stratagene, La Jolla, CA, U.S.A.), directionally cloned into a UniZAP XR vector phage (Stratagene), and packaged using the ZAP-cDNA Gigapack II Gold cloning system (Stratagene). Approximately 2 and 1.7 million recombinants were obtained for RC688^s and HD198^r cDNA libraries, respectively.

Development of probes

Lambda DNA of an amplified RC688^s cDNA library was prepared using phage precipitation and

phenol/chloroform extraction procedures after RNase A and DNase I digestions (Titus, 1991). Polymerase chain reaction (PCR) was carried out with a reverse vector primer T7 and a forward degenerate primer, 5'-TG(CT)CA(AG)GG(ACGT)GA(CT)(AT)(CG)(ACGT)GG(ACGT)GG(ACGT)CC(ACGT)(CT)T-3', designed from a highly conserved region (CQGDSGGPL) in both *M. sexta* trypsin and chymotrypsin cDNAs located approximately 250 bp from the 3' end (Peterson *et al.*, 1994, 1995). PCR amplified DNA fragments (~250 bp) were cloned into a TA vector (Promega, Madison, WI, U.S.A.). Nine clones were isolated and verified for insert size by PCR amplification and gel electrophoresis. Sequences of these clones were obtained through thermal cycle sequencing reactions, the products of which were visualized on silver-stained polyacrylamide gels (Promega).

cDNA library screening

The cDNA libraries were plated to a density of ~130 plaque forming units per cm², and plaques were transferred to nylon membranes (MSI, Westboro, MA, U.S.A.). The membranes were hybridized at 55°C (LabLogix DNA/RNA Hybridization Kit; LabLogix) with a PCR amplified chymotrypsin probe labelled with α -³²P-dCTP (Amersham, Arlington Heights, IL, U.S.A.). Nylon membranes were washed at 55°C for 1 h with three changes of 0.2 × SSC (sodium chloride/sodium citrate)/0.1% SDS and then exposed to X-ray films. Positive clones from the first library screen were subjected to PCR amplification and analysed for the presence of an insert fragment of the predicted size (~250 bp when amplified by degenerate and T7 priming, ~1.1 kb when amplified by T3 and T7 priming). Positive clones after PCR were plated to a density from which a single plaque could be readily obtained without cross contamination. PCR amplification was repeated to locate a single plaque with an insert of the predicted size from each clone identified with the ³²P-labelled probe. These positive clones were subcloned *in vivo* into pBlue-Script SK(±) phagemid (Stratagene). The cDNA inserts were sequenced by primer walking from both directions using thermal cycle sequencing (Promega) and an automated sequencer (Applied Biosystems model 393A, Foster City, CA) located at the College of Veterinary Medicine, Kansas State University, Manhattan, U.S.A.

Sequence analysis

The Blastx non-redundant database of the National Center for Biotechnology Information Internet server was used to perform similarity searching and the retrieval of homologous sequences (Altschul *et al.*, 1990; Gish and States, 1993). The Wisconsin Sequence Analysis Package GCG Unix version 8.1 (Genetics Computer Group, Madison, WI, U.S.A.) and sequence analysis tools of the SWISS-PROT Internet server were used to process data of deduced protein sequences. The sequences have been deposited in the GenBank database under the accession

numbers AF015611 for the Bt-susceptible strain and AF015610 for the Bt-resistant strain.

Chymotrypsin-like gene expression in gut tissue and non-gut tissue

To test for tissue-specific expression of the chymotrypsin-like protein gene, 200 fourth instar larvae from the RC688^s strain were used for the dissection of tissues. After removal of the anterior and posterior ends of the larvae, gut tissue was separated from the rest of the body. Total RNA was extracted and 10 µg of RNA from the gut tissue and the remaining body tissue minus the gut were used for Northern analysis (Ausubel *et al.*, 1994). RNA from gut tissue and non-gut tissue was subjected to 1% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane (MSI). A ~250 bp probe, PCR amplified from the clone closely related to chymotrypsinogen cDNA using vector primers T7 and SP6, was labelled with α -³²P-dCTP (Amersham) and used to hybridize target RNA on a nylon membrane at 55°C for approximately 16 h, using a DNA/RNA hybridization kit (LabLogix). The nylon membrane was washed at 55°C for 1 h with three changes of wash solution (0.2 × SSC and 0.1% SDS) and exposed to X-ray film for 24 h. After full sequences of the two chymotrypsinogen-like cDNAs from the cDNA libraries were obtained, the RNA expression levels probed using a partial fragment of the chymotrypsinogen-like cDNA were verified by rehybridizing the nylon membrane with a full-length chymotrypsinogen-like cDNA probe labelled with α -³²P-dCTP.

Southern blot analysis of genomic DNA from Bt-susceptible and Bt-resistant strains

Genomic DNA was extracted from fourth instar larvae using an isolation buffer containing 100 mM Tris-HCl (pH 9), 1% SDS, and 100 mM ethylene diamine tetraacetic acid (EDTA). Southern analysis was used to search for chymotrypsin gene differences between the *P. interpunctella* strains and the procedures followed are described by Southern (1975), Ausubel *et al.* (1994), and Wang *et al.* (1995). Three double restriction enzyme digestions, *Pst*I plus *Eco*RI, *Eco*RI plus *Hind*III, *Hind*III plus *Pst*I, and a control were conducted for each insect strain. In each digestion, 5 µg of genomic DNA was double-digested with 25 units of each enzyme for 5 h at 37°C. Digested DNA was separated using an 0.8% agarose gel, transferred onto a nylon membrane (MSI), and hybridized with a full-length chymotrypsinogen-like cDNA probe labelled with α -³²P-dCTP at 55°C for 20 h using a DNA/RNA hybridization kit (LabLogix). The nylon membrane was washed three times and exposed to X-ray film.

Chymotrypsin-like gene expression in Bt-susceptible and Bt-resistant strains

Chymotrypsin-like gene expression in fourth instar larvae of the two strains of *P. interpunctella* was analysed

by Northern blotting (Ausubel *et al.*, 1994). Five micrograms of mRNA from each strain was subjected to 1% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane (MSI). A ~250 bp probe, PCR amplified from the clone closely related to chymotrypsinogen cDNA using vector primers T7 and SP6 was labelled with biotin (Sigma). The target RNA was hybridized with the labelled probe at 50°C for approximately 16 h (LabLogix). The membrane was developed with a luminescent DNA/RNA detection kit (LabLogix) and exposed to X-ray film. To verify mRNA expression levels probed with a partial fragment of chymotrypsinogen-like cDNA, Northern blot analysis was repeated using a full-length chymotrypsinogen-like cDNA as a probe. Messenger RNA was purified from another group of larvae and the Northern blot was conducted with 3 µg of mRNA per lane. The mRNA was transferred to a nylon membrane after gel electrophoresis and hybridized with a full-length chymotrypsinogen-like cDNA probe labelled with α -³²P-dCTP at 55°C for 20 h. The nylon membrane was washed three times and exposed to X-ray film.

RESULTS

Chymotrypsin-like enzyme activity

The ability to hydrolyse SAAPFpNA, a chymotrypsin-diagnostic substrate, was previously reported to be substantially reduced in pooled gut extracts from an *entomocidus*-resistant strain of *P. interpunctella* (Oppert *et al.*, 1996). Using a microplate assay, decreased hydrolysis of SAAPFpNA was also observed in gut extracts from individual larvae of the Bt-resistant strain. The average specific activity (ΔA_{405} per min per mg of protein in the gut extract, $n = 16$ for each strain) of SAAPFpNA-hydrolysis obtained using gut extracts from the Bt-resistant strain was 9.6 ± 1.5 , which was approximately three-fold lower than that using extracts from the Bt-susceptible strain (25.1 ± 3.0).

Proteinase activity blots were used to identify SAAPFpNA-hydrolysing enzymes in individual larval gut extracts from the Bt-susceptible and Bt-resistant strains (Fig. 1). Five major bands of activity were observed in each larvae. However, when the intensity of diazotized nitroaniline was compared among individuals, the amount of activity in the faster migrating enzymes of the Bt-resistant individuals was greatly reduced. SAAPFpNA-hydrolysing proteinases were observed with apparent masses of approximately >250, 100, 34, 30, and 28 kDa. This difference in the chymotrypsin-like enzyme activity in the *P. interpunctella* strains indicated that resistance to Bt may be correlated with a decreased level of specific activity of chymotrypsin-like enzymes. The chymotrypsinogen-like enzyme mRNA levels and also the cDNA sequences in the Bt-susceptible and Bt-resistant strains of *P. interpunctella* were then investigated.

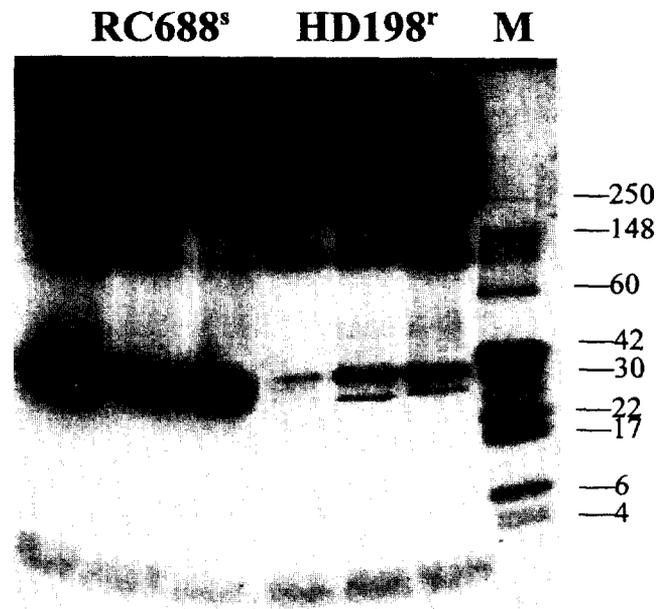


FIGURE 1. Proteinase activity blot of gut extracts from individual larvae of *P. interpunctella* strains RC688^s (Bt-susceptible) and HD198^r (Bt-resistant) incubated with N-succinyl-ala-ala-pro-phe p-nitroanilide (SAAPFpNA). M, molecular mass markers.

Probes generated with PCR amplification

Nine clones containing a fragment amplified from the RC688^s cDNA library were selected and subjected to plasmid DNA extraction and insert sequence analysis. Results of the GenBank Blastx homology search indicated that the sequences (~250 bp) of eight clones were similar to those of serine proteinases. All of these fragments contained a poly-A tail, indicating that the cloned fragments contained the 3'-end of the insect cDNAs. The clone with the highest homology to a chymotrypsinogen-like protein of *M. sexta* was PCR amplified with two vector primers, T7 and SP6. The PCR product was used as a probe for Northern analysis and cDNA library screening.

cDNA sequences

The cDNA libraries of RC688^s and HD198^r were screened with the chymotrypsin-like cDNA probe. Preliminary sequencing identified two clones from each cDNA library that had 3'-ends (~250 bp) identical to chymotrypsin-like cDNA sequences. One clone was selected from each library for sequencing and characterization.

A cDNA of a putative chymotrypsinogen-like proteinase was obtained from the RC688^s library, which contained 977 nucleotides with the start codon ATG at positions 50–52 and the termination codon TAG at positions 896–898 (Fig. 2). A clone obtained from the HD198^r library had 951 nucleotides and an open reading frame sequence identical to the cDNA from the RC688^s library. The open reading frame includes 846 nucleotides which encode 282 amino acid residues in a predicted immature form of a chymotrypsin-like proteinase. A polyadenyl-

S	GTCAATCACAGGATAGGTTAAGTCATCAATTCTTGAGCTAAAAATAAAG	49
R	*****	25
	ATGGCGTCAAAGATTCTGTTGTGCATACTGTTTGTGGGGTCCAAAGTGAAGTTTGTACT	109
	*****	85
	MetAlaSerLysIleLeuLeuCysIleLeuPheValGlyValGlnSerGluValLeuThr	20
	GTGCATAACTATCACATGAACATAGGAGTACCTCGTGCTATTAACCTGATGAACTCGGAA	169
	*****	145
	ValHisAsnTyrHisMetAsnIleGlyValProArgAlaIleAsnLeuMetAsnSerGlu	40
	TTGATGACTAGGATTGTCGGGGGAAGTCAAGTGACTACACCGACCTCATTTCGGTTTCAA	229
	*****	205
	LeuMetThrArg <u>IleValGlyGly</u> SerGlnValThrThrProThrSerPhePropheGln	60
	GCAGGTATAATAGCAACACTTACAACCTGGGTTTACCTCGATTTGTGGAGGTACACTCCTC	289
	*****	265
	AlaGlyIleIleAlaThrLeuThrThrGlyPheThrSerIleCysGlyGlyThrLeuLeu	80
	TCCAACACCAAGGTCCTAFACTGCGGCCACTGCTGGTGGGACGGCCAGAGCCAAGCCAGA	349
	*****	325
	SerAsnThrLysValLeuThrAlaAlaHisCysTrpTrpAspGlyGlnSerGlnAlaArg	100
	CTCTTACGGTGGTTCTGGGCTCCCTCACCATCTTCTCAGGAGGGACAAGGATAGAAACA	409
	*****	385
	LeuPheThrValValLeuGlySerLeuThrIlePheSerGlyGlyThrArgIleGluThr	120
	TCGAGAATAGTCGTTACCCCAACTGGAACACGAATGAGATCACCCATGATATTGCTATG	469
	*****	445
	SerArgIleValValHisProAsnTrpAsnThrAsnGluIleThrHisAspIleAlaMet	140
	GTGACTATTGCGAGAGTCAGTTTTACTAACAATATCCAGAGCATCCCTATACCTGACTTA	529
	*****	505
	ValThrIleAlaArgValSerPheThrAsnAsnIleGlnSerIleProIleProAspLeu	160
	GCAGATATCAATCACAACTTTGCCGGAGCATCGGCTGTGGTGTCCGGATACGGGAAAAC	589
	*****	565
	AlaAspIleAsnHisAsnPheAlaGlyAlaSerAlaValValSerGlyTyrGlyLysThr	180
	AGTGACGGACAAGGCAGCTTCCCAACCACGACATCGCTTACCAGACAACAGTCCAAGTG	649
	*****	625
	SerAspGlyGlnGlySerPheProThrThrThrSerLeuHisGlnThrThrValGlnVal	200
	ATCACCACCGCCGTTTGTGAGAAGAGTTTCGACATCACCTTCACGGGAGTCACCTGTGC	709
	*****	685
	IleThrAsnAlaValCysGlnLysSerPheAspIleThrLeuHisGlySerHisLeuCys	220
	ACCAACGGACAAGGTGGAGTGGGATCCTGTGACGGCGACTCGGGTGGACCCTTGACTACA	769
	*****	745
	ThrAsnGlyGlnGlyGlyValGlySerCysAspGlyAspSerGlyGlyProLeuThrThr	240
	ATTGAAACAATCGCAGAACTGTGATTGGAGTGGTGTGCTTTGGATTGGGTGACCGCTGT	829
	*****	805
	IleArgAsnAsnArgArgThrValIleGlyValValSerPheGlyLeuGlyAspArgCys	260
	CAGTCAGGCTACCCATCTGTCTACACCCGCGTTACTGCTTTCCTCACCTGGATCCAGGCA	889
	*****	865
	GlnSerGlyTyrProSerValTyrThrArgValThrAlaPheLeuThrTrpIleGlnAla	280
	AACTTGTAGAAAAATTCACCAAGAGCTGGGATTGCGAAATAGTCTGCATAAAAAATAAAAT	949
	*****G*****	925
	AsnLeu	282
	TAGCAAATATAAAAAAFAAAAAA	977
	*****	951

FIGURE 2. Nucleotide and deduced amino acid sequences of chymotrypsinogen-like cDNA isolated from *P. interpunctella*. S, clone from susceptible strain RC588; R, clone from resistant strain HD198. *Nucleotide identity between resistant and susceptible strains; ATG, start codon; TAG, termination codon; AATAA, polyadenylation signal; ▲, predicted signal peptide cleavage site; ◆, predicted activation peptide cleavage site. Conserved N-terminal residues Ile-Val-Gly-Gly are underlined. A 241 bp fragment used for Northern hybridization is located between two arrows → ← at the 3'-end of chymotrypsinogen-like cDNA.

ation signal, AATAA, was located at positions 941–945 in the RC688^s cDNA and at nucleotides 917–921 in HD198^r cDNA. Alignment of the two cDNA sequences showed only one nucleotide difference between the susceptible and resistant strains. At the 3'-end, a nucleotide change from adenine to guanine occurs in the resistant strain at position 940 just prior to the polyadenylation signal sequence.

Sequence comparison and evolutionary distance

A search of the GenBank Blastx non-redundant database revealed that the sequence of the *P. interpunctella* cDNA is similar to cDNAs in the serine proteinase trypsin/chymotrypsin family. Homologous sequences include elastase, collagenase, and other serine proteinases from several species of Lepidoptera, Coleoptera, and Diptera. The most similar sequences include chymotrypsin-like and elastase-like enzymes from *M. sexta* (Peterson and Wells, 1995; Peterson *et al.*, 1995), a serine protease (Moire *et al.*, 1994) and collagenase (Lecroisey *et al.*, 1987) from the common cattle grub, *Hypoderma lineatum* (Villers), a serine protease from the fruit fly, *Drosophila melanogaster* (Yun and Davis, 1989), a chymotrypsin-like enzyme from the blowfly, *Lucilia cuprina* (Casu *et al.*, 1994), and a serine proteinase from the malaria mosquito, *Anopheles gambiae* (Siden-Kiamos *et al.*, 1996). Using the GCG GAP Comparison software to compare the *P. interpunctella* chymotrypsin-like protein sequence to protein sequences of seven other insect species (the signal sequence was excluded), the predicted mature chymotrypsin of *P. interpunctella* is most similar to the chymotrypsin-like enzyme of *M. sexta* with 68% similarity and 52% identity in the amino acid sequences (Table 1). GCG Evolutionary Distance analysis also revealed that the chymotrypsinogen-like protein from *P. interpunctella* is most similar to the *M. sexta* chymotrypsin-like protein (Table 1).

The predicted amino acid sequence encoded by the *P. interpunctella* cDNA was aligned with seven homologous insect proteinases using the GCG Pileup program (Fig. 3). This sequence contains all of the conserved residues in the putative active site, His⁹⁰, Asp¹³⁷, and Ser²³⁴, which form the catalytic triad or "charge relay system"

in serine proteases (Wang *et al.*, 1993; Peterson *et al.*, 1994). Six cysteine residues, predicted to occur in disulfide bridge configurations among trypsins and chymotrypsins, are located at positions 75, 91, 206, 220, 230, and 260, respectively. The same pattern of cysteine residues is found in all seven insect serine proteases. The deduced amino acid sequences of the pre-chymotrypsinogen-like proteins from both the susceptible and resistant *P. interpunctella* cDNA libraries contain a predicted signal peptide 16 amino acid residues long and a 28 amino acid activation peptide (SWISS-PROT, Sequence Analysis Tools). The calculated molecular weights for the precursor of chymotrypsinogen and the mature enzyme are 30,021 and 25,081 Da, respectively, which are similar in size to the *P. interpunctella* gut chymotrypsin-like proteins separated by SDS-PAGE (Oppert *et al.*, 1996). The sequence IVGG is highly conserved in many trypsin- and chymotrypsin-like proteins and marks the N-termini of the active enzymes (Wang *et al.*, 1993). Although we do not have amino acid sequence data, it is predicted that the mature chymotrypsin-like enzymes of *P. interpunctella* corresponding to this cDNA sequence contain 238 amino acid residues and have amino termini of "IVGG", which are identical to the N-terminal sequence of the *M. sexta* chymotrypsin-like protein that contains 234 amino acid residues (Peterson *et al.*, 1995). The putative chymotrypsin-like proteinase would correspond to one of the faster migrating, 28–34 kDa SAAPFpNA-hydrolysing proteinases (Fig. 1), which exhibits reduced activity in the Bt-resistant strain of *P. interpunctella*.

Chymotrypsin-like gene expression in gut and non-gut tissues

Northern analysis of RNAs isolated from gut tissue and non-gut tissue indicated that proteinase cDNAs isolated from whole body tissues were primarily from the digestive tract. Chymotrypsinogen-like RNA from gut tissue (Fig. 4, lane 2 in panels A and B) exhibited substantially higher expression levels than RNA from non-gut tissues (Fig. 4, lane 1 in panels A and B). This result demonstrates that the chymotrypsin-like gene is expressed predominantly in the gut.

TABLE 1. Similarity comparison and evolutionary distance relationship (GCG: Kimura Protein Distance) of a *P. interpunctella* chymotrypsinogen-like protein to serine proteinase-like enzymes from seven other insect species

Species	Proteinase type	Similarity (%)	Identity (%)	Evolutionary distance
<i>H. lineatum</i>	Collagenase	57	37	
<i>L. cuprina</i>	Chymotrypsin	58	38	
<i>M. sexta</i>	Chymotrypsin	68	52	
<i>P. interpunctella</i>	Chymotrypsin	–	–	
<i>M. sexta</i>	Elastase	63	46	
<i>A. gambiae</i>	Serine proteinase	55	34	
<i>D. melanogaster</i>	Serine proteinase	55	33	
<i>H. lineatum</i>	Serine proteinase	57	37	

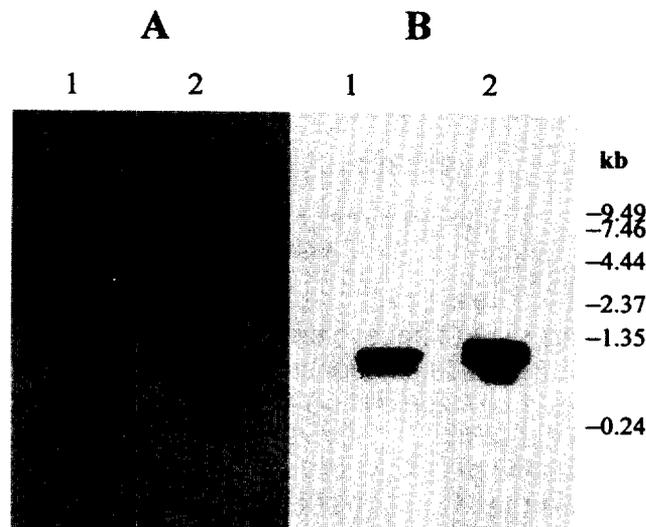


FIGURE 4. Northern analysis of chymotrypsinogen-like RNA expression levels in *P. interpunctella* larval body tissue without gut (lane 1) and in gut tissue (lane 2). Ten micrograms of total RNA per lane from each tissue was subjected to 1% agarose/formaldehyde gel electrophoresis, and transferred to a nylon membrane. RNA was hybridized with ^{32}P -dCTP labelled probe at 55°C, and the membrane was washed for 1 h with three changes of solution. (A) RNA was hybridized with a ~250 bp cDNA probe cloned using a degenerate primer and a vector primer binding to a region from the 3'-end of chymotrypsinogen-like cDNA. (B) RNA was hybridized with a full length of chymotrypsinogen-like cDNA.

Southern blot analysis of genomic DNA from *Bt*-susceptible and *Bt*-resistant strains

Southern analysis was performed to estimate the number of chymotrypsin gene copies in the genomes of *Bt*-susceptible and *Bt*-resistant strains of *P. interpunctella* (Fig. 5). The susceptible and resistant chymotrypsinogen-like cDNAs were examined, but no restriction sites in either cDNA were present for the three enzymes used, *Pst*I, *Eco*RI and *Hind*III. The Southern blot shows that one major fragment (~20 kb) and two minor fragments (~15 and 25 kb) were present in the *Pst*I/*Eco*RI digest, and only two fragments (~4 and ~17 kb) were observed in both the *Eco*RI/*Hind*III and *Hind*III/*Pst*I digests (Fig. 5). These restriction sites are probably located either in introns of the chymotrypsin-like gene or fragments outside of the exons. On the basis of the results of both Southern and Northern blot analyses (see next section), there appears to be more than one copy of the chymotrypsin-like gene in the *P. interpunctella* genome. Both strains have the same number and sizes of fragments, which indicates that the genomic organization for the chymotrypsin-like genes in the two strains is similar.

Chymotrypsin-like gene expression in *Bt*-susceptible and *Bt*-resistant strains

To examine whether chymotrypsin-like gene expression is the same in the two *P. interpunctella* strains, Northern blots containing mRNAs from RC688^r and HD198^r were hybridized with a PCR-generated probe. The data showed that the chymotrypsinogen-like

cDNA probe hybridized to two different sizes of mRNAs and yielded fragment sizes of ~1 and ~3.3 kb (Fig. 6). The 1 kb fragment corresponded in size to the cloned chymotrypsinogen-like cDNAs. The 3.3 kb fragment, although not consistently present in all Northern blots, may represent a second gene with similarity to the 1 kb chymotrypsinogen-like cDNA. This result supports the hypothesis that more than one copy of the chymotrypsin-like gene exists in *P. interpunctella* as was indicated previously by Southern analysis (Fig. 5).

DISCUSSION

Activities of enzymes in gut extracts from individual larvae of the *entomocidus*-resistant strain were lower when assayed using a chymotrypsin-diagnostic substrate, SAAPFpNA, relative to those from larvae of the *Bt*-susceptible strain. This difference was observed using two different enzyme assay methods, one a spectrophotometric assay using p -nitroanilide conjugated substrates and the other a proteinase blot assay that distinguishes both the number and relative molecular masses of SAAPFpNA-hydrolysing enzymes. There were five distinct SAAPFpNA-hydrolysing activities in gut extracts from both strains, with several proteinases of approximately 28–34, 100, and over 250 kDa in mass. The activities of the 28–34 kDa enzymes were lower in the *Bt*-resistant strains, whereas the levels of the 100 and over 250 kDa enzymes appeared similar to those found in gut extracts from the *Bt*-susceptible strain. Therefore, the 28–34 kDa enzymes are more likely to be associated with resistance to *Bt* toxins than the other proteinases.

Serine proteinases are present in the insect alimentary tract for the hydrolysis of proteins in food. Wang *et al.* (1995) demonstrated that trypsin-like gene expression in *C. fumiferana* was tissue specific and only occurred in digestive tissues. No RNA expression from non-digestive tissues of *C. fumiferana* was detected. In *M. sexta* chymotrypsin mRNA was expressed more in the anterior and middle portions of the midgut, whereas trypsin-like mRNA was higher in the middle and posterior sections (Peterson *et al.*, 1995). Tissue-specific expression of digestive proteinases was also observed in the European corn borer, *Ostrinia nubilalis* (Hübner) by Houseman and Chin (1995) and in *D. melanogaster* by Matsumoto *et al.* (1995).

Although the expression level of the chymotrypsinogen-like RNA is low in non-gut tissues, this low level may represent true expression in non-gut tissues. Chymotrypsinogen-like cDNA of *P. interpunctella* is most similar to *M. sexta* gut chymotrypsin-like cDNA (Peterson *et al.*, 1995). The presence of chymotrypsin-like enzymes in the gut, the conserved N-terminal amino acid sequence, the predicted protein size from *P. interpunctella* cDNA, and the predominant expression of chymotrypsinogen-like RNA in gut tissues are consistent with the hypothesis that chymotrypsinogen-like gene expression in *P. interpunctella* occurs in the gut. The low

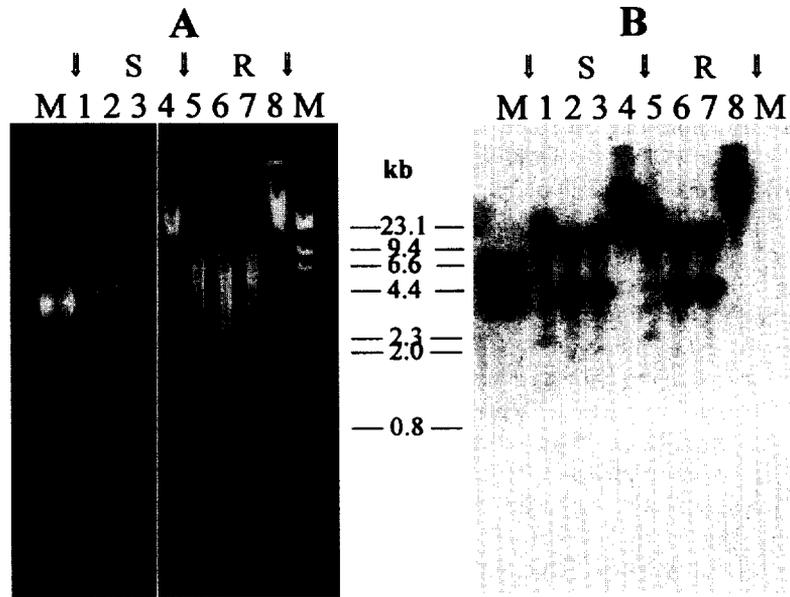


FIGURE 5. Southern analysis and comparison of genomic DNA from Bt-susceptible strain RC688^s (lanes 1–4) and Bt-resistant strain HD198^r (lanes 5–8) of *P. interpunctella*. S, susceptible and R, resistant. (A) Ethidium bromide stained gel; 5 μ g/lane of double-digested DNA was separated on 0.8% agarose gel; enzymes used for lanes 1 and 5, *Pst*I + *Eco*RI; lanes 2 and 6, *Eco*RI + *Hind*III; lanes 3 and 7, *Hind*III + *Pst*I; lanes 4 and 8, undigested control DNA; M, DNA marker (left marker was 100 bp ladder from Pharmacia and right marker was high-range ladder from Bio-Rad). (B) Autoradiograph of Southern analysis; lanes are the same as those denoted in panel A; digested DNA was transferred to nylon and hybridized with a full-length chymotrypsinogen-like cDNA labelled with ³²P-dCTP.

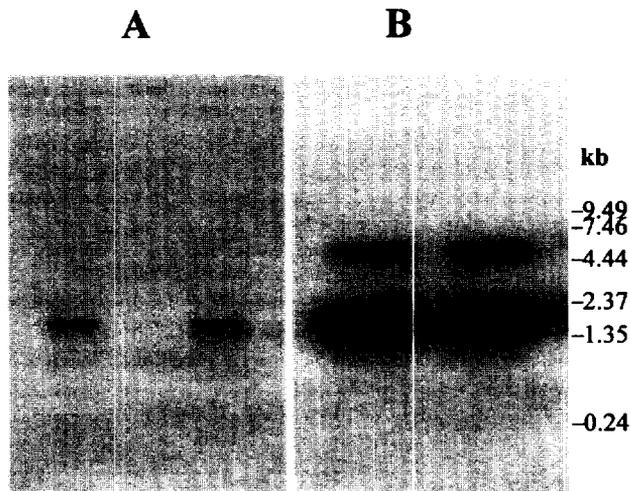


FIGURE 6. Comparison of chymotrypsinogen-like mRNA expression levels in two *P. interpunctella* strains. Lane 1, Bt-susceptible strain RC688^s; lane 2, Bt-resistant strain HD198^r. (A) 5 μ g of mRNA per lane from each strain was subjected to 1% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane. mRNA was hybridized with a ~250 bp cDNA probe cloned using a degenerate primer and a vector primer binding to a region at the 3'-end of chymotrypsinogen-like cDNA and labelled with biotin. (B) 3 μ g of mRNA from each strain was loaded in each lane. mRNA was hybridized with a full-length chymotrypsinogen-like cDNA labelled with ³²P-dCTP.

level of RNA expression found in the body tissue other than the gut may have resulted from contamination with gut tissues during dissection, which was difficult to avoid due to the small size of these larvae. These data demonstrate that the tissue specificity of chymotrypsinogen-like

gene expression in *P. interpunctella* is similar to that of chymotrypsin and trypsin-like gene expression in other lepidopteran insects.

Susceptibility of late instar larvae to Bt toxin declines slightly relative to that of earlier instars (McGaughey, 1978). A reduced susceptibility to Bt toxin as a function of age also was observed in other insect species, but this relationship may also be influenced by ecological characteristics of the insect (Liu *et al.*, 1995), or increased activity and diversity of gut proteases in the late instars, which are involved in degrading proteins (Keller *et al.*, 1996). Although the Bt-susceptible and Bt-resistant strains of *P. interpunctella* exhibit different levels of proteinase activity (Oppert *et al.*, 1996), the mRNAs (~1 kb band, Fig. 6) encoding a chymotrypsin-like proteinase are expressed in RC688^s and HD198^r at similar levels during the fourth instar. Therefore, the lower activity of the chymotrypsin-like proteinase in the Bt-resistant strain could be due to an altered yield or stability of gene products controlled by post-transcriptional regulatory factors, or changes in gut proteinase profiles and specificity influenced by developmental regulating factors.

The expression of proteinases in insects is regulated by developmental processes and dietary components. Chapman (1985) suggested that short-term regulation of enzymatic activity is consistent with a secretagogue mechanism and longer-term regulation is probably due to a hormonally based mechanism. During insect metamorphosis, trypsin-like protein expression is transcriptionally regulated by juvenile hormone titer in the cabbage looper, *Trichoplusia ni* (Hübner) (Jones *et al.*,

1993). The regulation of trypsin-like mRNA abundance in *T. ni* occurs at both the transcriptional and turnover levels. Transcription of this trypsin-like mRNA is suppressed and delayed if juvenile hormone does not decline as normal, and the mRNA becomes less stable after commitment to the pupal molt (Jones *et al.*, 1993).

In most insects, the midgut cells synthesize and secrete digestive enzymes in response to the presence of protein in the midgut (Shambaugh, 1954). Felix *et al.* (1991) suggested that post-feeding induction of trypsin activity in *A. aegypti* is a two-phase process regulated at the midgut cellular level. The first phase of trypsin synthesis is stimulated by soluble proteins of variable sizes and involves translation of messenger RNA already available within the midgut cells. The second phase is stimulated by small peptides and requires complete synthesis of new mRNA from DNA. Borovsky *et al.* (1996) provided further evidence that the trypsin gene in liver-fed *Neobellieria bullata* is translationally controlled by trypsin-modulating oostatic factors.

It is unclear how *P. interpunctella* larvae regulate gut proteinase expression and activity. Unlike blood-feeding dipterans, lepidopterans take several meals a day. The Bt-resistant strain used in this study has been maintained on the artificial diet supplemented with a dose of 125 mg Bt/kg for 110 generations, whereas the susceptible strain has been maintained on a diet not treated with Bt. The similar chymotrypsin-like mRNA expression levels in these two insect strains suggest that transcriptional regulation is not correlated with the difference in chymotrypsin activity in these two strains. To understand how the Bt-resistant strain modulates its chymotrypsin-like activity, further experiments need to be carried out to investigate mRNA stability, translational regulation, and the involvement of factors affecting stability and activity of the enzyme itself.

In summary, we have cloned and sequenced cDNAs for a chymotrypsinogen-like protein from Bt-susceptible and Bt-resistant strains of *P. interpunctella*. The open reading frames are identical and encode a mature protein that is similar in sequence to several serine proteinases from other insect species. Levels of mRNA are similar in the Bt-susceptible and Bt-resistant strains. The data demonstrate that the coding regions of the genes are the same, and suggest that transcription of the chymotrypsinogen-like gene is not correlated to insect resistance to Bt. Because levels of the chymotrypsin-like enzyme activity in the guts of the two strains is quite different, translation of the mRNAs or post-translational modification and processing of the proteins may be different, or other chymotrypsin genes not recognized by our probes may be involved in insect resistance to Bt.

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