



Analysis of midgut proteinases from *Bacillus thuringiensis*-susceptible and -resistant *Heliothis virescens* (Lepidoptera: Noctuidae)

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Abstract

Insects with altered proteinases can avoid intoxication by *Bacillus thuringiensis* (Bt) toxins. Therefore, proteinase activities from gut extracts of Bt-susceptible (YDK) and -resistant (YHD2-B, CXC and KCBhyb) *Heliothis virescens* strains were compared. The overall pH of gut extracts from YDK and CXC were statistically similar (9.56 and 9.62, respectively), while the pH of extracts from KCBhyb and YHD2-B were significantly more alkaline (9.81 and 10.0, respectively). Gut extracts from YHD2-B and CXC larvae processed Cry1Ac and Cry2Aa protoxin slower than extracts from YDK larvae, suggesting that differences in proteolysis contribute to resistance in these strains. Casein zymogram analysis of gut extracts revealed both qualitative and quantitative differences in caseinolytic activities among all strains, but the overall caseinolytic activity of YHD2-B gut extract was lower. Kinetic microplate assays with a trypsin substrate (L-BApNA) demonstrated that proteinases in YDK gut extract had increased alkaline pH optima compared to resistant strains YHD2-B, CXC and KCBhyb. Gut extracts from YHD2-B had reduced trypsin-like activity, and activity blots indicated that YHD2-B had lost a trypsin-like proteinase activity. In assays with a chymotrypsin substrate (SAAPFpNA), enzymes from all Bt-resistant strains had increased pH optima, especially those from KCBhyb. Activity blots indicated that CXC had lost a chymotrypsin-like proteinase activity. Because serine proteinases are a critical component of Bt toxin mode of action, these differences may contribute to decreased toxicity in the Bt-resistant strains.

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1. Introduction

Heliothis virescens, the tobacco budworm, is a major pest of cotton in the United States, and is currently controlled by cotton plants engineered to express *Bacillus thuringiensis* Cry1Ac toxin. Second generation Bt cotton plants expressing Cry1Ac and Cry2Ab toxins display increased budworm control as well as improved control of alternative lepidopteran pests (Chitkowski et al., 2003). However, the continued success of Bt

cotton depends on methods to detect and delay insect resistance to Cry proteins (Ferre and Van Rie, 2002).

When susceptible lepidopteran larvae ingest Bt crystals, the crystals dissolve in the alkaline lumen of the midgut, releasing the Cry protoxin form. Released protoxin is cleaved by midgut proteinases to a stable toxin core (Lightwood et al., 2000) that is then capable of binding to membrane-associated receptor proteins. Toxin receptor binding leads to oligomerization of toxin molecules, which results in binding to secondary receptors, including proteins and glycolipids, and toxin insertion into the epithelial membrane, forming pores that culminate in insect death (Schnepf et al., 1998; Bravo et al., 2004; Griffiths et al., 2005).

The role of proteinases in insect resistance to Bt Cry toxins is complex due to the distinct routes by which proteinases may affect toxin function. Loss of a specific midgut trypsin in *Plodia*

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Table 1
Toxicity, resistance ratios and resistance factors in Bt-susceptible and -resistant strains of *H. virescens*

Strain	Selection toxin	LC ₅₀ ^a		Resistance ratio ^b		Resistance factors		Slower processing of protoxin ^c	
		Cry1Ac	Cry2Aa	Cry1Ac	Cry2Aa	HevCaLP ^d	¹²⁵ I-Cry1Ac binding ^e	Cry1Ac	Cry2Aa
YDK	None	0.73	14.5	NA	NA	+	+	–	–
YHD2-B	Cry1Ac	>2000	138	73,700	9.5	–	–	+	+
CXC	Cry2Aa	211	>1000	290	>250	+	+	+	+
KCBhyb	Cry2Aa	137	>1000	188	>250	–	+	–	–

^a LC₅₀ values are expressed as µg of toxin/ml of diet (Kota et al., 1999; Jurat-Fuentes et al., 2002, 2003).

^b Resistance ratio = LC₅₀ of the resistant strain/LC₅₀ of YDK (Kota et al., 1999; Jurat-Fuentes et al., 2002, 2003). NA = Not Applicable.

^c Slower rate of protoxin processing as observed in this study.

^d The expression or absence of HevCaLP is indicated by + or – (Jurat-Fuentes et al., 2004).

^e BBMV binding assays (Jurat-Fuentes et al., 2002, 2003).

interpunctella, necessary for converting protoxin to toxin, accounted for partial resistance to Cry1A toxins (Oppert et al., 1997). A similar proteinase-related Bt resistance mechanism was reported for Bt-resistant *Ostrinia nubilalis* (Li et al., 2004). In both insects, resistance was almost completely reversed when insects were fed activated toxin (Herrero et al., 2001; Li et al., 2005), providing compelling evidence that activation of protoxin is compromised in these insects. Resistance also may be associated with degradation and elimination of toxin (Forcada et al., 1996). Additional examples of proteinase-related Bt resistance have been reported for the coleopterans *Melolontha melolontha* and *Leptinotarsa decemlineata* (Loseva et al., 2002; Wagner et al., 2002).

The Bt-resistant strains of *H. virescens* analyzed in this study are listed in Table 1. After selection with Cry1Ac crystals, the YHD2 strain (the precursor to YHD2-B) had become highly resistant to Cry1Ac, had lost Cry1Aa binding, and yet retained Cry1Ab and Cry1Ac binding (Lee et al., 1995). This resistant phenotype was accounted for by retrotansposon-mediated disruption of a single gene, *BtR-4*, that encodes the HevCaLP cadherin protein. HevCaLP was predicted to function as a toxin receptor, and the genetic alteration in resistant insects resulted in a form of HevCaLP that lacked transmembrane and toxin binding domains (Gahan et al., 2001). Jurat-Fuentes et al. (2004) confirmed that “knock-out” of *BtR-4* resulted in loss of Cry1Aa, but not Cry1Ab or Cry1Ac binding. Continued selection of YHD2 larvae with Cry1Ac resulted in strain YHD2-B, which is >73,000-fold resistant to Cry1Ac toxin (Jurat-Fuentes et al., 2002), one of the largest reported resistance levels to Bt in insects. Additional mutations or adaptive events in YHD2-B have led to the loss of Cry1Ab and Cry1Ac binding (Jurat-Fuentes et al., 2004). Reduced toxin binding and increased resistance in YHD2-B correlated with reduced levels of a specific membrane-bound alkaline phosphatase in larvae from this strain (Jurat-Fuentes and Adang, 2004).

The Cry1Ac and Cry2Aa resistant KCBhyb and CXC strains were generated from the *H. virescens* Bt-resistant strains KCB and CP73, respectively, by selection with diet containing Cry2Aa inclusion bodies. Loss of HevCaLP accounted for Cry1Ac resistance in KCBhyb, but not in CXC larvae (Jurat-Fuentes et al., 2004). Although the resistance mechanism for CXC is unknown (Jurat-Fuentes et al., 2003), the parental CP73 strain, which was resistant to Cry1Ac and Cry2Aa, exhibited

slower protoxin processing and faster toxin degradation (Forcada et al., 1996). Unfortunately, the CP73 strain no longer exists, and comparisons with this strain are not possible.

The goal of this study was to investigate the potential role of proteinase alteration in resistance to Cry toxins in these strains. We analyzed the profiles of digestive proteinases in Bt-resistant strains of *H. virescens*, including YHD2-B, KCBhyb, and CXC, relative to Bt-susceptible YDK larvae. Analyses included a time-course study of Bt protoxin activation, casein zymograms, kinetic microplate assays, and proteinase activity blots using trypsin and chymotrypsin specific substrates. Specific differences in serine proteinases, both quantitative and qualitative, were detected for the resistant strains compared to the susceptible strain. The relevance of these results to Bt-resistance management is discussed.

2. Materials and methods

2.1. Insect strains

H. virescens larvae were kindly supplied by Dr. Fred Gould (North Carolina State University, Table 1). The Bt-susceptible strain, YDK, was the parental strain for selection of the YHD2 strain on artificial diet containing Cry1Ac protoxin (MVP, Dow AgroSciences, Indianapolis, IN), that resulted in 10,000-fold resistance to Cry1Ac (Gould et al., 1995). Continuous selection of this strain with Cry1Ac has led to strain YHD2-B, with increased levels of Cry1Ac resistance (>73,000-fold) (Jurat-Fuentes et al., 2002). The CXC strain, previously referred to as CPN X CP73-3 (Forcada et al., 1999), is about 289-fold resistant to Cry1Ac (Jurat-Fuentes et al., 2003) and resulted from the mating of Cry1Ac resistant CP73-3 adults with susceptible insects and further selection on Cry2Aa protoxin produced in *E. coli* (Kota et al., 1999). Strain KCBhyb resulted from mating Cry1Ac resistant-KCB adults (Forcada et al., 1999) with susceptible moths and selection on Cry2Aa (Fred Gould, unpublished data), and is 187-fold resistant to Cry1Ac (Jurat-Fuentes et al., 2003). Both CXC and KCBhyb have high levels of resistance to Cry2Aa, and no mortality was observed with doses up to 1000 µg/ml of toxin (Jurat-Fuentes et al., 2003). All insect strains were reared on artificial diet, under laboratory conditions as described previously (Gould et al., 1995).

2.2. Determination of midgut lumen pH

Fourth instar larvae were chilled on ice and dissected between the 3rd and 4th prolegs. Larvae were held upright so as to prevent loss of midgut fluids. A pH microelectrode (Model M41410, Microelectrodes Inc. Bedford, NH) was inserted into the gut lumen and the pH value recorded. Measurements were taken from the midguts of five larvae from each strain. A one-way analysis of variance was conducted using the Fisher LSD (Systat Software, Inc., Point Richmond, CA).

2.3. Preparation of gut extracts

Midguts from five late 4th instar larvae of each strain were dissected, five dissected guts pooled in 25 μ l of sterile H₂O, and briefly vortexed. One gut equivalent (GE) was equal to 5 μ l of sample. The samples were centrifuged at 13,000 \times g for 15 min at 4 $^{\circ}$ C, and the supernatant was carefully separated from the pellet. Protein in the supernatant, referred to subsequently as gut extract, was quantified by a dye binding assay (Bradford, 1976) and aliquots were stored at -80° C. Each GE contained approximately 100 μ g of protein.

2.4. Protoxin and toxin purification

Bt strain HD-73 producing Cry1Ac toxin was obtained from the *Bacillus* Genetic Stock Collection (Columbus, OH). Cry1Ac toxin was purified as described elsewhere (Luo et al., 1999).

An *Escherichia coli* strain harboring the *Cry2Aa* gene was obtained from Dr. Donald H. Dean (Ohio State University, Columbus, OH). Bacteria were cultured in LB medium containing 100 μ g/ml ampicillin for three days at 37 $^{\circ}$ C.

Bacterial cells were pelleted by centrifugation, the pellet was resuspended in lysis buffer (15% sucrose, 50 mM EDTA, 50 mM Tris pH 8.0, 10 μ g/ml of lysozyme) and Cry2Aa protoxin was purified according to methods previously described (Audtho et al., 1999). Purified toxin was quantified using a dye binding assay (Bradford, 1976) and aliquots were stored at -80° C.

2.5. Temporal processing of Cry1Ac and Cry2Aa protoxin

Proteolytic processing of Cry1Ac and Cry2Aa protoxin was evaluated by incubating 10 μ g of purified protoxin with gut extract (total protein was 40 μ g) in a final volume of 30 μ l of pH 9.75 universal buffer (Frugoni, 1957) at room temperature for increasing periods of time. Proteolysis was terminated by heating samples at 100 $^{\circ}$ C for 5 min. Samples were cooled, loading buffer was added, and the samples were again heated at 100 $^{\circ}$ C for 5 min (Oppert et al., 1994). Samples were subjected to electrophoresis in 10% Tris–Glycine gels (Bio-Rad) and stained with Coomassie blue. Gels shown are representative of five independent assays.

To test the significance of the protoxin activation differences between strains, we quantified the intensity of the resultant toxin band in the gels shown in Fig. 1. Band intensities corresponding to that of processed toxin were measured using the Alpha Ease FC Densitometry software on the FluorChem™ IS-8900 imaging system (Alpha Innotech Inc.). Band intensity, which is represented by the area under the associated peak, was measured for each of the five replicates and the corresponding values fed into Sigma Stat 3.1 statistical software. One-way analysis of variance was conducted using the Fischer LSD and Tukey tests. When band alterations when compared to patterns of extracts from susceptible larvae were

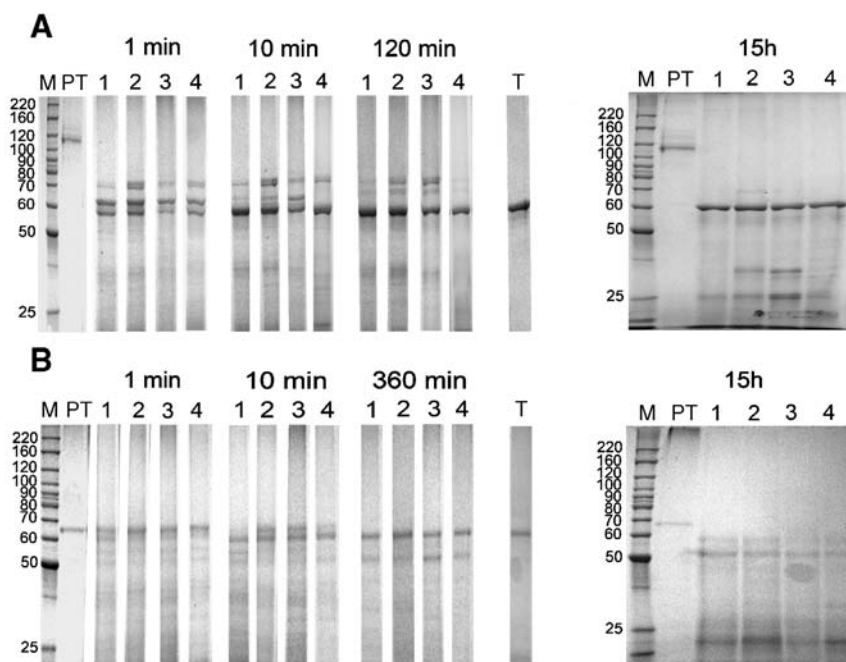


Fig. 1. Proteolytic digestion of Cry1Ac (Panel A.) and Cry2Aa (Panel B) protoxins by gut extracts from larvae of Bt-susceptible YDK (lane 1) and -resistant YHD2-B (lane 2), CXC (lane 3) and KCBhyb (lane 4) *H. virescens* larvae. PT = Purified protoxin; T = purified trypsin activated toxin.

Table 2

Comparison of midgut lumen pH values in Bt-susceptible (YDK) and Bt-resistant (YHD2-B, CXC, and KCBhyb) *H. virescens* strains

Strain	Mean pH±S.D. ^a
YDK	9.62±0.15 a
YHD2-B	10.0±0.17 b
CXC	9.56±0.06 a
KCBhyb	9.81±0.16 b

^aComparisons based on triplicate measurements from five individual larvae from each strain (mean±standard deviation). Means with different letters are statistically different as determined by Fisher LSD ($p<0.05$).

detected, the differences were statistically significant at the $P<0.001$ level.

Toxin band intensities in the gels as measured by densitometry were used to calculate the rate of toxin activation by gut extracts of the susceptible and resistant strains. Protoxin input (10 µg) was used to calculate the amount of toxin activated per minute for each sample. Because the majority of Cry1Ac toxin is activated within the first minute, and Cry2Aa is processed within 10 min of incubation, we used these time frames to calculate the corresponding toxin activation rates for each strain.

2.6. Casein zymogram analysis

Gut extracts (total protein was 85 µg) were separated by electrophoresis in 10% Tris–Tricine gels at 4 °C (Schagger, 1987). After electrophoresis, the gel was incubated overnight at 4 °C in 50 mM Tris–HCl buffer (pH 7.5) containing 2% casein, followed by incubation at room temperature for 20 min (Garcia-Carreno et al., 1993). After Coomassie staining, clear bands of proteinase activity were visible against a dark blue background.

2.7. Microplate assays

The enzymatic activity in gut extracts (40 µg total protein) from each strain was analyzed in a 96-well microplate assay (Oppert et al., 1997), in triplicate, using in buffers of increasing pH (Frugoni, 1957). The microplate was preincubated in a microplate reader for 2 min at 37 °C for temperature equilibration. To initiate the reaction, 50 µl of N_α -Benzoyl-DL-arginine p -nitroanilide (L-BAPNA) or N -succinyl-Ala-Ala-Pro-phenylalanine p -nitroanilide (SAAPFpNA) (2.2 mM final concentration) substrate solution was added, and absorbance was measured at 405 nm. Readings were taken at 11 s intervals for 5 min at 37 °C, during which time all increases in absorbance were linear. SigmaPlot software was used to calculate standard errors and plot graphs.

2.8. Activity blots

To compare the number and relative activities of gut proteinases, gut extracts (85 µg total protein) from each strain were resolved in a 10% Tris–Tricine gel. Electrophoresis conditions were as for casein zymograms. Proteins were transferred to nitrocellulose membranes by electroblotting and

then membranes were incubated in 200 mM Tris pH 9.75, 20 mM CaCl_2 containing 2.2 mM L-BAPNA or SAAPFpNA at 37 °C for 20 min (Oppert et al., 1996). The incubation was at pH 8.0 rather than the optimal pH for gut enzyme activity because of auto-hydrolysis of the substrate in higher pH buffers, resulting in indistinguishable bands of activity due to the higher background. Following the appearance of yellow bands of released nitroaniline, the product was diazotized to visualize activity bands, and membranes were placed in sealed plastic bags and stored at –20 °C until archived by photodocumentation.

3. Results

3.1. pH of *H. virescens* larval midgut fluid

Because Bt crystals are dissolved in the midgut lumen and activated by proteinases, factors in the midgut lumen are critical to toxin action. Therefore, the pH of midgut contents was measured for larvae from Bt-susceptible and resistant strains. The lumen pH was similar for YDK and CXC, 9.56 and 9.62, respectively. However, the gut pH was significantly more alkaline for KCBhyb and YHD2-B, measuring 9.81 and 10.0, respectively (Table 2). There was no significant difference in the midgut pH values of YHD2-B and KCBhyb.

3.2. Protoxin activation by *H. virescens* gut extracts

A study of protoxin hydrolysis was conducted to determine if there were differences in protoxin activation by gut extracts from the different *H. virescens* strains. Cry1Ac and Cry2Aa protoxins were incubated with gut extracts for increasing periods of time, and products were analyzed by SDS-PAGE.

Enzymes in all gut extracts digested Cry1Ac protoxin through 74-, 72-, and 64-kDa intermediates, to a presumably active ~58-kDa toxin (Fig. 1A). Most of the activation process was observed to occur within 1 min of incubation. In this time

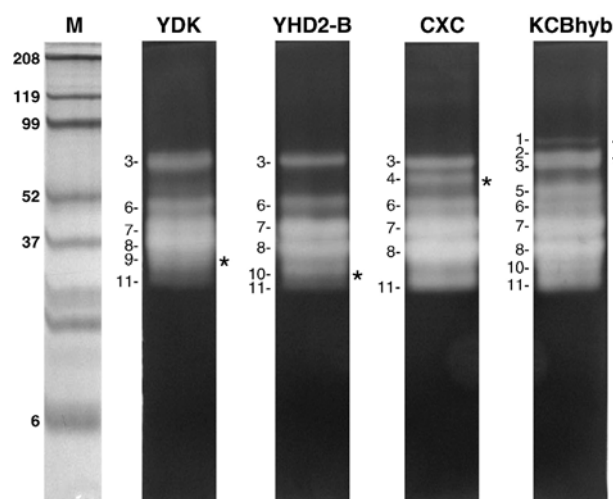


Fig. 2. Casein zymogram of gut extracts from YDK, YHD2-B, CXC and KCBhyb. Relative caseinolytic activities are numbered from slower to faster migrating on the left side of each sample, and unique activities are marked on the right with an asterisk. M: molecular mass markers.

frame, gut extract from CXC larvae activated toxin more slowly (0.90 $\mu\text{g}/\text{min}$) than extracts from YDK larvae (1.23 $\mu\text{g}/\text{min}$). Gut extracts from YHD2-B (1.26 $\mu\text{g}/\text{min}$) had a rate of toxin activation similar to YDK extracts, while KCBhyb extracts displayed faster activation rates (2.01 $\mu\text{g}/\text{min}$). However, enzymes in extracts from resistant strains YHD2-B and CXC had less effective toxin activation than extracts from YDK larvae as evidenced by the presence of increased amounts of intermediates after 120 min of incubation when compared to YDK or KCBhyb extracts. A ~ 64 kDa intermediate remained in incubations with YHD2-B extracts after 15 h. Toxin degradation products of ~ 40 and 25 kDa were found in incubations with YHD2-B and CXC enzymes, and only the 25 kDa product was apparent in incubations with YDK and KCBhyb after 15 h. There was no apparent further proteolysis of the ~ 58 kDa form in extracts from any of the strains after longer incubation times (data not shown).

Similar results were observed when Cry2Aa protoxin was incubated with *H. virescens* gut extracts (Fig. 1B). Enzymes from the susceptible YDK and the resistant KCBhyb larvae hydrolyzed the ~ 68 kDa protoxin to a 60 kDa intermediate form at the same rate (0.34 $\mu\text{g}/\text{min}$). Further proteolysis to a 55 kDa apparent toxin peptide, and a 48 kDa presumably non-toxic Cry2Aa peptide (Audtho et al., 1999), also was detected in the extracts. Gut extracts from resistant strains YHD2-B and CXC processed Cry2Aa protoxin at slower rates (0.30 mg/min and 0.28 mg/min respectively), than YDK or KCBhyb extracts, as an intermediate was observed only in these reactions after 10 min. Traces of protoxin were detected even after a 30 min incubation of Cry2Aa with YHD2-B gut extract (data not shown). All extracts produced detectable amounts of the presumably inactive 48 kDa Cry2Aa peptide. After 15 h, toxin degradation products of ~ 25 and 20 kDa were found in all samples, with an additional ~ 40 kDa product observed only in incubations with enzymes from KCBhyb larvae.

3.3. Zymogram analysis of gut extracts

Because protoxin hydrolysis experiments suggested differences between strains in protoxin processing, proteinases in each strain were compared. A zymogram analysis was used to assess the relative number of soluble gut proteinases in Bt-susceptible and -resistant larvae. There were at least 11 caseinolytic activities discernable in extracts from the four strains (Fig. 2). However, there were subtle differences in both quantitative and qualitative proteinase activities in gut extracts from the different strains. Although the overall activity patterns were similar, enzymes in YDK, CXC, and KCBhyb gut extracts demonstrated an increased caseinolytic activity relative to that of YHD2-B. Certain activities (bands 3, 6, 7, 8, and 11) were found in all extracts. KCBhyb had the most distinct caseinolytic activities, with unique activities (bands 1, 2, and 5) that were not found in the other extracts. Other unique activities included band 9, only found in YDK, band 4, only found in CXC, and band 10, only found in YHD2-B and KCBhyb.

3.4. Determination of trypsin- and chymotrypsin-like proteinase activities at various pH

Serine proteinases are prevalent in lepidopteran larvae and critical enzymes in the conversion of Bt protoxin to toxin. Therefore, substrates were used to evaluate the activities of typical serine proteinases in extracts from the four *H. virescens* strains. The pH optima for trypsin- and chymotrypsin-like enzymes in gut extracts were determined by the hydrolysis of L-BAPNA and SAAPFpNA, respectively, using a kinetic microplate assay. These substrates were not hydrolyzed in acidic buffers by enzymes in gut extracts from either susceptible or resistant strains (Fig. 3). Rates of hydrolysis of both substrates increased in buffers of pH greater than 6, with maximum hydrolysis detected between pH 8–11.2. At pH 9.6, the gut pH of larvae from strains YDK and CXC, the rate of BAPNA hydrolysis by gut enzymes was greater than for KCBhyb and YHD2-B gut enzymes at their physiological pH, 9.9 (Fig. 3A). In assays with a chymotrypsin substrate (SAAPFpNA), enzymes in KCBhyb extracts at their physiological pH were

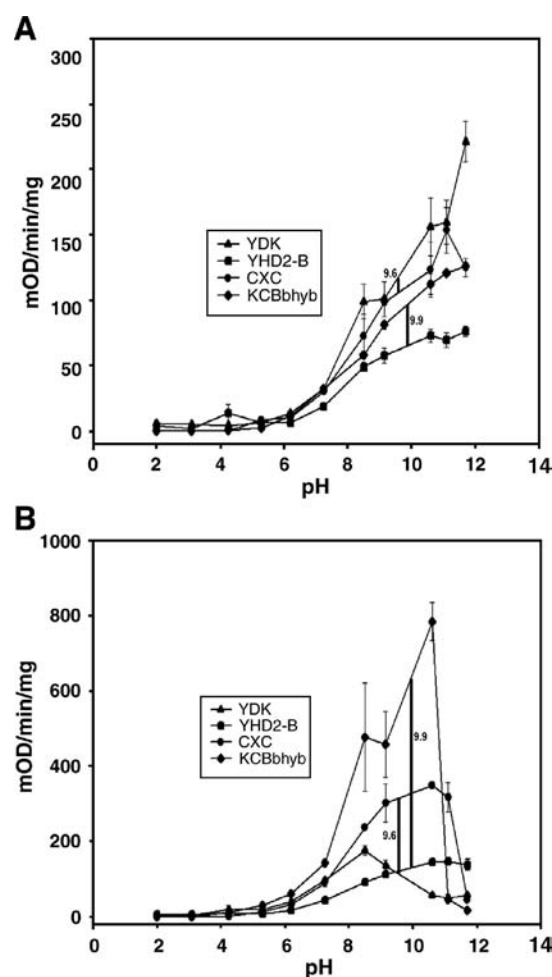


Fig. 3. Activities of trypsin- and chymotrypsin-like gut proteinases from YDK, YHD2-B, CXC and KCBhyb larvae with L-BAPNA (A) and SAAPFpNA (B), respectively, in buffers of increasing pH. A bar denoting the average physiological pH of the gut of YDK and CXC (9.6) and YHD2-B and KCBhyb (9.9) is noted on each graph.

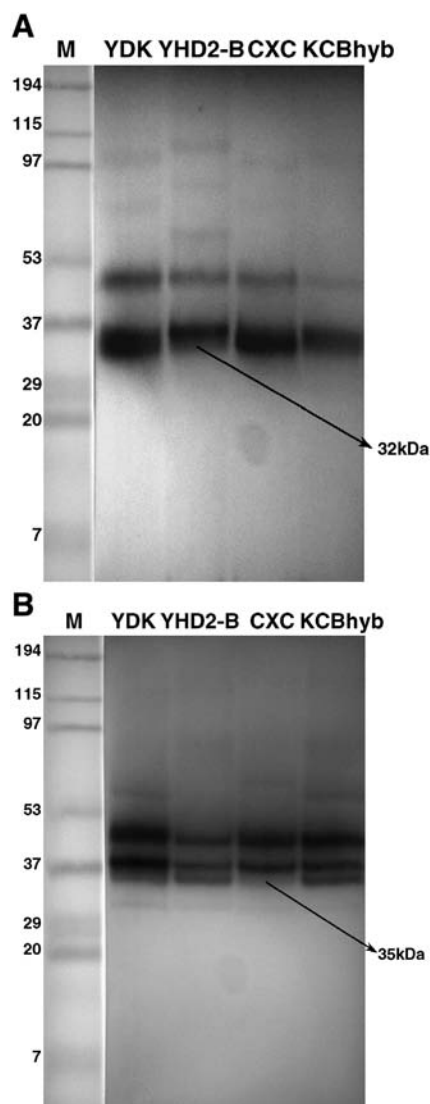


Fig. 4. Activity blot analysis of trypsin- and chymotrypsin-like proteinases from YDK, YHD2-B, CXC and KCBhyb larvae using L-BAPNA (A) and SAAPFpNA (B), respectively. Faint or missing activities are denoted on the right.

approximately 2–4 times more active than enzymes from the other gut extracts, with optimal activity at pH 10.6, but sharply reduced at pH 11.2 (Fig. 3B). In comparison, the chymotrypsin-like activity of CXC enzymes at pH 9.6 was intermediate, whereas the activity of enzymes from YHD2-B at pH 9.9 and YDK at pH 9.6 was much lower.

3.5. Activity blot assays for trypsin- and chymotrypsin-like proteinases

To test whether the quantitative differences in trypsin and chymotrypsin activities detected in the microplate assays were due to the gain or loss of specific proteinases, we performed activity blot analyses to study the molecular masses of trypsin- and chymotrypsin-like proteinases in gut extracts from all strains. Blots incubated in pH 8.0 buffer containing L-BAPNA revealed similar patterns of trypsin-like enzymes in gut extracts

from all four strains, with proteinases of ~48 and ~34 kDa common to all extracts (Fig. 4A). Weaker intensity bands were detected at ~100, ~98, ~97, ~77, and ~62 kDa, and may have resulted from protein aggregation in the extracts under the pH conditions used for electrophoresis. However, a trypsin-like proteinase of ~32 kDa was observed in YDK, CXC and KCBhyb extracts but was not detected in gut extracts from YHD2-B larvae. Activity blots with SAAPFpNA revealed that the most active chymotrypsin-like proteinases common to all strains were ~35, 37, and 47 kDa in mass (Fig. 4B). All strains had weak chymotrypsin-like activity at ~33 kDa. The activity of the 35 kDa band was barely detectable in CXC extracts, relative to extracts from the other strains. Additionally, faint bands of chymotrypsin-like activity were detected at ~66 and 73 kDa only in extracts from YDK and KCBhyb larvae.

4. Discussion

In this study, gut proteinases from larvae of Bt-susceptible and resistant *H. virescens* strains were examined for the relative hydrolysis of Cry1Ac protoxin, Cry2Aa protoxin, and casein. Since trypsin and chymotrypsins are the two major classes of proteinases in this insect species (Johnston et al., 1995), and because these enzymes are involved in the activation of Cry protoxins (Rukmini et al., 2000), we examined gut extracts from larvae of the susceptible and resistant strains for differences in activities of these enzymes. Additionally, we compared the gut pH in larvae from all strains, because gut pH determines protein solubility, enzymatic activity and is a factor in toxin susceptibility. All of these studies identified differences among the strains that may contribute to reduced susceptibility to Bt toxins.

Larvae from the YHD2-B strain do not express the full length HevCaLP cadherin, a key Cry1A toxin receptor in *H. virescens* (Gahan et al., 2001; Jurat-Fuentes et al., 2004). While reduced amounts of a Cry1Ac binding alkaline phosphatase probably contribute to resistance (Jurat-Fuentes and Adang, 2004), our results suggest that altered proteinase levels also should be considered as a potential resistance factor in this strain. The reduced rate of protoxin processing by YHD2-B extracts is consistent with the reduced activity of trypsin-like proteinases and the absence of a ~32 kDa trypsin-like protein. A similar reduction of specific trypsin-like proteinases was reported for Bt-resistant strains of *P. interpunctella* and *O. nubilalis* (Oppert et al., 1996; Li et al., 2004). Our results with YHD2-B larvae and published studies with other insect species support the contention that reduction of trypsin-like proteinases is an adaptive mechanism in lepidopteran larvae that delays processing of protoxin to toxin and, in this way, confers some degree of resistance to Bt crystals and protoxin. Certainly, insects have adaptive capacity to plant proteinase inhibitors and may incorporate this strategy for survival of other toxins. However, the relative contribution of proteinase alterations to resistance in this strain remains to be determined.

Mechanisms of altered toxin activation are generally associated with low levels of resistance (Ferre and Van Rie, 2002). In a Bt-resistant strain of *P. interpunctella*, the alteration

of gut proteinases accounted for ~90% of the resistance (Herrero et al., 2001). The CXC strain of *H. virescens* is resistant to Cry1Ac and Cry2Aa toxins, yet the resistance has not been explained by reduced Cry1A toxin binding (Jurat-Fuentes et al., 2003). Therefore, the relative contribution of proteinase-mediated resistance in this strain may be more important than in YHD2-B. Previously, (Forcada et al., 1999) reported altered proteinase patterns in gut extracts from CXC, as well as enhanced midgut epithelium recovery after toxin challenge, compared to susceptible controls. Continuous selection with diet containing Cry2Aa toxin resulted in higher levels of resistance to this toxin in the CXC strain (Jurat-Fuentes et al., 2003). In our assays, we detected reduced levels of a 35 kDa chymotrypsin-like proteinase activity in gut extracts from CXC larvae when compared to extracts from larvae in other strains. We believe this protein is the same as the 36 kDa proteinase absent in gut extracts from CXC larvae in the previous study (Forcada et al., 1999). The elevated chymotrypsin activity levels detected in enzymes from gut extracts from this strain may represent compensatory alterations in the gut to counterbalance the reduced levels of the 35 kDa proteinase. In our study, extracts from CXC larvae processed Cry1Ac protoxin at a slower rate than YDK extracts, with protoxin intermediates remaining after the longest digestion time. A similar phenomenon was reported in the parental CP73-3 strain, which processed protoxin more slowly (Forcada et al., 1996). Those authors also reported that CP73-3 extracts degraded toxin more rapidly than a control susceptible strain. We were unable to confirm this observation in our experiments.

With respect to trypsin-like activity, CXC gut extracts had activities similar to YDK extracts in both kinetic and activity blot assays. While these results seem internally contradictory, it is important to consider that the activity blots provide qualitative estimations, while the kinetic assays present quantitative data. The CXC extracts also contained a 66 kDa protein with caseinolytic activity (band 4, Fig. 2) that was not detected in the trypsin or chymotrypsin activity blots. Possibly, this 66 kDa protein represented a different type of proteinase that was not detected by the ρ -nitroanilide substrates. Overall, the CXC larvae had reduced amounts of a 35 kDa chymotrypsin-like enzyme, a reduced capacity to process both Cry1Ac and Cry2Aa protoxin, and an increased capacity to degrade Cry2Aa toxin. These results are consistent with previous reports on the proteinases of CXC and one of its parental strains (Forcada et al., 1996, 1999). These data suggest that after continuous selection with Cry2Aa, proteinase alterations were conserved, indirect evidence that these alterations may correlate with resistance against Cry1Ac and Cry2Aa.

The KCBhyb larvae are characterized by Cry1Ac and Cry2Aa resistance and loss of Cry1Aa binding (Jurat-Fuentes et al., 2003). Even though the retrotransposon-mediated knock-out was detected in this strain, this allele is not fixed in KCBhyb (Jurat-Fuentes et al., 2004). Forcada et al. (1999) reported reduced levels of 34 and 44 kDa proteinases in gut extracts from larvae of the KCB strain, one of the parental strains for the KCBhyb strain. Chymotrypsin-like activity was much greater in KCBhyb gut extracts compared to extracts from susceptible

larvae, and zymogram analysis detected several additional caseinolytic proteinases unique for KCBhyb extracts. Whether these proteinases have a role in Cry1Ac or Cry2Aa resistance is yet to be determined. However, no differences in protoxin activation or toxin degradation were observed in this strain when compared to YDK protoxin activation patterns.

Under natural conditions, the diversity of trypsin and chymotrypsin isoforms in gut extracts of lepidopteran larvae contribute to digestion of a complex plant diet and protect insects from harmful effects of plant resistance proteins. In *H. virescens*, selection for resistance to Bt Cry1Ac and Cry2Aa protoxins (for CXC and KCBhyb larvae) has resulted in resistant larvae with different qualitative and quantitative proteinase patterns. Additional research is needed to characterize the specific altered proteinases in Bt-resistant insects and investigate their potential role in resistance to Bt toxins.

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Appendix A

L-BAPNA- N_{α} -benzoyl-L-Arginine- ρ -nitroanilide; SAAPFP NA- N -succinyl-Ala-Ala-Pro-Phe- ρ -nitroanilide; Cry — Crystal; Bt — *Bacillus thuringiensis*; HevCaLP — *Heliothis virescens* cadherin like protein; EDTA — ethylenediamine-tetraacetic acid; LB — Luria Bertani.

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