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Research

Activity and expression of midgut proteases from Mexican and US *Trichoplusia ni* (Hübner) strains exposed to *Bacillus thuringiensis*

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Abstract

Proteases in the insect midgut have been associated with differences in susceptibility to *Bacillus thuringiensis* (Bt) insecticidal toxins. Therefore, we evaluated the protease activity of laboratory versus field strains of *Trichoplusia ni* that were previously characterized by their susceptibility to Bt Cry toxins or protoxins and antimicrobial peptide production (Tamez-Guerra et al. 2006, 2008). In addition, because aminopeptidases may be involved in Bt resistance, the expression of *T. ni* aminopeptidase N1 transcript (*tnapn1*) was analyzed by RT-PCR. These strains included Mexican (NL) and American (US) laboratory strains, as well as a field collected strain (GTO), and strains obtained by XenTari® selection from the laboratory strains (NLX and USX) or field strain (GTOX). The activity of proteases in midgut extracts were evaluated by in vitro assays or in-gel activity with specific substrates. The results indicated that the ratio of midgut protease activity (trypsin:chymotrypsin) was higher in the laboratory NL and US strains, but the differences did not correlate with Bt resistance. The ratio was slightly increased in the Xentari-selected field strain GTOX, and zymograms with Xentari or Cry1Ac as substrates suggested that both high and low molecular mass protease activities were increased in GTOX. Selection also resulted in 87% and 300% higher *tnapn1* amplification in Xentari-selected NLX and USX, respectively. The relationship between Bt susceptibility, proteases and *tnapn1* expression and activity alterations is discussed.

Key Words: trypsin; chymotrypsin; Bt protoxins and toxins activation; aminopeptidase N1; enzymatic alterations

Resumen

Diferencias en la susceptibilidad de insectos a *Bacillus thuringiensis* (Bt) se han asociado a la actividad de proteasas intestinales del insecto. El presente estudio se realizó para evaluar la actividad de proteasas del intestino medio de cepas de *Trichoplusia ni* de laboratorio y campo en las que anteriormente se había caracterizado su susceptibilidad a protoxinas y toxinas Cry de Bt y producción de péptidos antimicrobiales (Tamez-Guerra et al. 2006, 2008). Debido a que la aminopeptidasa N1 se ha relacionado con la baja de susceptibilidad a Bt, también se analizó la amplificación de este transcrito (*tnapn1*) por RT-PCR entre las cepas de *T. ni*. Las cepas evaluadas fueron de laboratorio de México (NL) y Estados Unidos (US), así como las cepas colectadas de campo (GTO) o después de sobreexponer las larvas a XenTari® (NLX, USX y GTOX). La actividad de proteasas presentes en el extracto del intestino medio se evaluó en ensayos in vitro o en gel usando sustratos específicos. Los resultados mostraron que el rango entre la relación de tripsina:quimotripsina era mayor en las cepas de laboratorio NL y US, pero estas diferencias no mostraron correlación con la resistencia a Bt. El rango se incrementó ligeramente al seleccionar la cepa de campo GTOX con XenTari, mientras que los zimogramas usando Cry1Ac o XenTari como sustrato sugieren que se incrementó la actividad de proteasas de alta y baja masa molecular, especialmente en la cepa GTOX. También se observó un incremento del 87% y 300% en la expresión del *tnapn1* en las cepas NLX y USX, respectivamente, seleccionadas con XenTari. Se discute la relación entre la susceptibilidad a Bt con la alteración de la expresión de *tnapn1* y la actividad de proteasas.

Palabras Clave: tripsina; quimiotripsina; activación de protoxinas y toxinas de Bt; aminopeptidasa N1; alteraciones enzimáticas

In the Bajío Guanajatense region in Mexico, *Bacillus thuringiensis* (Bt) Berliner, is one of the approved bioinsecticides in integrated pest management (IPM) programs. Bt activity against insects relies on Cry toxins production. Cry protoxin structures have been documented where a Cry 3-domain protein family with similar mode of action, including Cry1Aa, was grouped. This 3-domain comprises domain I (*N*-terminal), responsible for toxin membrane insertion and pore-formation, whereas domains II and III are involved in receptor binding. Most of the Cry protoxins activation involves the proteolytic removal of the *N*-terminal peptide; then, the activated toxin binds to specific receptors in the midgut before inserting into the membrane. In lepidopteran larvae, aminopeptidase N (APN) is one of at least 4 Cry1A protein-binding toxins (Bravo et al. 2007).

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Bt-insecticides have been applied on cruciferous crops, among others; however, Bt resistance has been observed in field populations of *Plutella xylostella* L. (Lepidoptera: Plutellidae). Partial resistance to the specific Cry toxins Cry1Ac and Cry1C, and the commercial products Dipel 2X® (Bt *kurstaki*), XenTari® (Bt *aizawai*), and Agree® (Bt *kurstaki* + *aizawai*) was reported for Mexican *P. xylostella* strains (Díaz-Gómez et al. 2000). In addition, partial resistance to Cry1Ab protoxin and toxin in a field-collected *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) strain also was detected, likely due to the common practice of using Bt products to control both pests on the same crops (Tamez-Guerra et al. 2006). *Plutella xylostella* and *T. ni* field-collected strains had higher immune responses related to increased antimicrobial peptide transcript expression compared to that of the laboratory strain (Tamez-Guerra et al. 2008).

Trichoplusia ni populations with resistance to Bt have been reported in the greenhouse (Janmaat & Myers 2003; Wang et al. 2007; Zhang et al. 2012; Kain et al. 2015). A Cry1Ac greenhouse-evolved resistant population of *T. ni* was crossed with a highly inbred susceptible laboratory strain, and progeny had increased midgut protease and hemolymph melanization activities (Wang et al. 2007). *Trichoplusia ni* resistance to Cry1Ac toxin in this strain was related to aminopeptidase N (APN1 and APN6) genes by a trans-regulatory mechanism, where APN1 was down-regulated and APN6 was up-regulated (Zhang et al. 2012; Tiewsiri & Wang 2011). Another resistance gene was mapped to the locus of an ABCC2 transporter gene (Park et al. 2014).

Resistance to Bt by laboratory-selected or greenhouse *T. ni* populations suffered fitness costs that induced a rapid decline of resistant populations (Janmaat & Myers 2003; Tamez-Guerra et al. 2006; Janmaat et al. 2014; Shikano & Cory 2014). The mechanisms behind the fitness cost among *T. ni* populations resistant to Bt remains unknown. Bt-resistant *T. ni* demonstrated more efficient conversion of nutrients than the susceptible strain under certain dietary conditions, and the LC_{50} decreased when resistant larvae were fed high levels of protein, suggesting changes in midgut proteases (Shikano & Cory 2014). Similar observations were recently reported by Deans et al. (2017) after testing *Helicoverpa zea* larval susceptibility against Cry1Ac, artificially feeding on different ratios of proteins and carbohydrates.

Primary protein digestion in lepidopteran larvae relies mostly on trypsin- and chymotrypsin-like serine protease activities. The functional diversity of digestive enzymes can be correlated with the adaptation of insects to host plants, as well as to exposure of insects to naturally occurring antagonistic biomolecules (Srinivasan et al. 2006). González-Cabrera et al. (2013) detected lower trypsin-, chymotrypsin-, and elastase-like activities in Bt-maize resistant *Mythimna unipuncta* (Haworth [Lepidoptera: Noctuidae]) larvae. Because Bt protoxins must be partially digested by proteolytic enzymes to be active, alterations in enzymatic activity can reduce larvae susceptibility to Bt (Oppert et al. 1994, 1996, 1997; Forcada et al. 1996), in addition to Cry protein receptor binding alterations, such as what has been reported with APN. The present study was undertaken to evaluate the differences in gut proteolytic activity and APN-1 (TnANP1) among *T. ni* strains with different susceptibilities to Bt protoxins, toxins, commercial products, and Bt crops.

Materials and Methods

INSECTS

Unless otherwise specified, reagents were from Sigma-Aldrich Química, Sociedad de Responsabilidad Limitada de Capital Variable (S. de R.L. de C.V.), Toluca, México.*Trichoplusia ni* larvae populations tested in this study originated from the same colonies, as previously reported (Tamez-Guerra et al. 2006). In brief, *T. ni* strains included a Mexican laboratory strain (NL) reared in our laboratory for 6 years, originally provided by Dr. Howard T. Dulmage (USDA-ARS, Weslaco, Texas, USA); an American laboratory strain (US), kindly provided by Dr. Robert W. Behle from the National Center for Agriculture Utilization Research (NCAUR), of the USDA-ARS, Peoria, Illinois, USA); and strains collected from San Luis de la Paz (GTO), located in Guanajuato State, Mexico (Table 1), collected from broccoli, cabbage, and cauliflower fields from Nov 2001 to May 2003. Insect colonies were reared on artificial diet (McGuire et al. 1997; Tamez-Guerra et al. 1998) at 25 °C ± 2 °C, 55 ± 10% relative humidity, and 16:8 h L:D photoperiod. When larvae reached the pupal stage, about 30 females and 25 males were pooled to initiate the colony. After 2 to 3 generations, *T. ni* colonies were separated into unexposed and exposed to XenTari, identified with the addition of a "X" to the name, where differences in susceptibility to Bt Cry toxins were observed (Tamez-Guerra et al. 2006, Table 2).

ENZYME ASSAYS

Trichoplusia ni larval midgut was obtained to evaluate trypsin and chymotrypsin activities from crude gut extracts. Twelve 4th instar (9-d-old) *T. ni* larvae were immobilized on ice and dissected by removing anterior and posterior tips of the larvae in ice-cold 50 mM Tris-HCl buffer (pH 8.0). The midgut with food bolus content plus the peritrophic membrane were removed gently from larvae, frozen immediately, and stored a −20 °C until use. To extract and determine the amount of protein for the enzymatic assays, samples were homogenized using a PRO-250 homogenizer (PRO Scientific Inc., Monroe, Connecticut, USA), with 5 ml of buffer (50 mM Tris-HCl, pH 8.0) per g of sample at 4 °C. The homogenate was centrifuged for 5 m at 12,000 g at 4 °C. The resulting pellet was discarded and the supernatant was processed for immediate protein analysis, using the Bio-Rad assay and bovine serum albumin as standard (Bradford 1976).

Trypsin and chymotrypsin activities were measured in vitro using the chromogenic synthetic substrates for trypsin and chymotrypsin, *N*-a-benzoyl-l-arginine-ρ-nitroanilide (BA*p*NA) and *N*-succinyl-ala-ala-pro-phe-ρ-nitroanilide (SAAPF*p*NA), respectively. Substrate stocks and buffers were thermally equilibrated at 25 °C before the assay. Reactions were initiated by adding 50 µl of 12 mM substrate in 50% dimethyl sulfoxide to 550 µl of 0.1 M Tris-HCl buffer, pH 8.0, containing 5 µl of the enzyme solution. Enzyme activities were determined by measuring optical densities at 405 nm (A_{405}) using 1 mM SAAPF*p*NA or BA*p*NA as substrates. The linear increase in A_{405} due to *p*-nitroaniline production was measured by continuous reading (every 10 sec) for 5 min, using a

Table 1. *Trichoplusia ni* strains used in this study.

Source	Country	Source	Code
Laboratory	USA/Mexico	UANL-FCB, Inmunología	NL
	USA	NCAUR-USDA-ARS, Peoria, Illinois	US
Field	Mexico	San Luis de la Paz, GTO	GTO
Selected	Mexico	GTO strain, selected with XenTari	GTOX
	USA	US strain, selected with XenTari	USX

Table 2. Response of *Trichoplusia ni* neonates exposed to *Bacillus thuringiensis* in an overlay bioassay. Data are the LC_{50} (CI_{95}); ND – not determined[1].

Strain	XenTari	Lepinox	Cry1Aa protoxin	Cry1Aa toxin	Cry1Ab protoxin	Cry1Ab toxin	Cry1Ac protoxin	Cry1Ac toxin
NL	1.17 (0.88-1.46)	1.01 (0.25-2.1)	54.7 (13.5-4,920)	6.92 (2.6-32.3)	0.7 (0.29-8.89)	1.6 (0.6-22.1)	0.1 (0.048-0.18)	0.4 (0.13-4.76)
NLX	ND	ND	1.6 (0.49-3.54)	2.0 (0.7-4.6)	2.2 (0.7-8.78)	0.5 (0.27-1.0)	0.7 (0.29-1.88)	2.1 (0.62-27.7)
US	1.54 (0.86-2.04)	ND	0.6 (0.30-0.89)	4.15 (1.2-43.1)	2.0 (0.8-22.1)	0.5 (0.24-0.97)	0.4 (0.14-1.01)	0.7 (0.24-3.54)
USX	ND	ND	2.7 (0.94-41.5)	2.4 (0.88-5.58)	2.4 (1.41-7.99)	1.0 (0.41-20.3)	2.1 (0.62-27.7)	0.07 (0.02-0.21)
GTO	1.03 (0.90-1.27)	1.92 (1.04-2.73)	ND	ND	ND	ND	ND	ND
GTOX	ND	ND	9.3 (4.35-44.1)	5.28 (3.97-36.2)	34.9 (18.0-70.1)	20.2 (8.4-43.8)	8.7 (5.18-50.1)	0.8 (0.3-2.4)

[1] Data adapted from Tamez-Guerra et al. (2006). LC_{50} is the concentration (ng x $10^2/cm^2$) resulting in 50% mortality; CI_{95} = 95% confidence intervals. Average from 4 replications, testing 12 neonates per dose.

Beckman spectrophotometer DU 650 (Beckman Coulter de México, Sociedad Anónima de Cantidad Variable (S.A. de C.V.), Ciudad de México).

Because sodium dodecylsulphate (SDS) can alter enzyme activity but is a component of gel electrophoresis, the effect of 0.1% and 1.0% SDS on azocasein hydrolysis by *T. ni* midgut proteases was evaluated. The biological lumen extracts and commercial enzymes were evaluated in a test tube assay using 3 SDS concentration percentages: 0.0, 0.1, and 1.0. Hydrolysis by commercial trypsin and chymotrypsin and enzymes in midgut extracts from each strain was evaluated by spectrophotometry at 404 nm, using a test tube assay with 1% azocasein as substrate and 10 m incubation at room temperature, as described by García-Carreño et al. (1993).

ZYMOGRAPHY

Substrate hydrolysis tests were performed using XenTari, Cry1Ac (HD-73) protoxin, or casein incubated in buffers, as well as casein that was gel-incorporated, to evaluate the proteolytic activity in *T. ni* larval midgut extracts, according to García-Carreño et al. (1993). Enzymes in midgut extracts of each *T. ni* strain (5 µl total protein, unexposed or XenTari-exposed) were separated by 10-20% Tris-tricine SDS-PAGE using an OWL P8DS electrophoretic chamber (Owl Separation Systems, Portsmouth, New Hampshire, USA), that allowed the samples to be separated under cooled (4 °C) conditions. For substrate-incorporated gels, 5.0 µg of total gut protein were analyzed in 4-16% Tris-glycine gels containing stained casein (ZBC, Invitrogen, Carlsbad, California, USA). For gels that were incubated post-electrophoresis with substrate, the same amount of total gut protein was used from each strain. Each sample was mixed with an equal volume of double concentrated (2X) sample buffer (0.125 mM Tris-hydrochloride pH 6.8, 20% glycerol, 4% SDS, and 0.005% bromophenol blue) and incubated for 10 m at room temperature. Separations were at a constant 35 mA and 4 °C. After electrophoresis, the SDS-gel was placed in 0.1 M TRIS buffer, pH 8.0. Gels without substrate were incubated with either 1% casein, 2% Xentari, or 1% Cry1Ac protoxin in TRIS buffer. All gels were incubated for 90 m for digestion of protein substrate by the active fractions at room temperature, washed with distilled water, and immediately fixed and stained in a 1-step process by placing them in a staining solution containing 40% ethanol, 10% acetic acid, and 0.1% Coomassie brilliant blue R-250. Clear zones on blue background indicated protease activity.

ACTIVITY BLOTS AND GELS

Activity blots were performed as previously described by Oppert et al. (1996). Trypsin, chymotrypsin, and elastase activities in extracts of 5 mg total protein from larval guts of laboratory (NL, US), field (GTO), and Xentari-selected (USX) strains were compared. Samples were mixed with loading buffer, and were subjected to electrophoresis in precast 12% Tris-glycine SDS polyacrylamide gels (BioRad, BIORAD S.A., Ciudad de México) at a constant 35 mA at 4 °C, using a Hoefer mighty small 250 (SE260 Mighty Small II Deluxe Mini Vertical Electrophoresis Unit, Hoefer Scientific, San Francisco, California). Pre-stained markers (Multimark, Invitrogen, Carlsbad, California, USA) were included to estimate the relative molecular masses of proteases. After electrophoresis, gels were transferred to nitrocellulose membranes and were incubated in 1 ml of 200 mM Tris-HCl, pH 8.0, 20 mM $CaCl_2$, and containing 0.5 mg per ml of BA*p*NA for trypsin, SAAPF*p*NA for chymotrypsin, and N-succinyl-ala-ala-pro-leu-*p*NA (SAAPL*p*NA) for elastase activity for 1 h at room temperature with gentle agitation. Released nitroaniline was diazotized by subsequent incubation of 5 m each in 0.1% sodium nitrite in 1 M HCl, 0.5% ammonium sulphamate in 1 M HCl, and 0.05% *N*-(1-naphthyl)-ethylendiamine in 47.5% ethanol. Membranes were stored at −20 °C. All enzymatic activity assays were performed in triplicate, using larval strains from different generations.

TRICHOPLUSIA NI APN1 AMPLIFICATION

Because the lack of *T. ni* aminopeptidase N1 (*tnapn1*) gene expression has been related to *T. ni* resistance to Bt, we used semi-quantitative RT-PCR to compare the *tnapn1* transcript abundance in the *T. ni* strains in this study (Wang et al. 2005). Tested *T. ni* strains were NL, US, and GTO, or XenTari-selected USX and NLX and GTOX (with 5 generations exposed to XenTari). Thirty neonates of USX and NLX and GTOX were exposed to the XenTari-calculated LC_{50} (150 IU per mL) in blue-stained 2% sugar solution using a droplet feeding bioassay dose, whereas NL, US and GTO were exposed to the same solution with no XenTari (Tamez-Guerra et al. 2006). Stained larvae were transferred to artificial diet and incubated at 25 °C ± 2 °C, 55 ± 10% relative humidity, and a 16:8 h L:D photoperiod for up to 7 d. Five surviving larvae were transferred to an empty cup to stop feeding overnight, and pooled midguts were analyzed. Total RNA was isolated from 5 µg RNA midgut mixture and was converted to cDNA with a high capacity cDNA reverse transcription kit (Applied Biosystem de México, S.A. de C.V., Ciudad de México). Primers to the constitutive ribosomal protein S5 (*rs5*) transcript as positive control (GenBank: AY837869.1) and *tnapn1* (GenBank: AY836579.1) were designed (Table 3). For each sample, 1 µL cDNA and 0.2 µM primer were added to Taq & Go (MP, Biomedicals, LLC, Santa Ana, California, USA). Transcripts were amplified for 30 cycles in a thermocycler (Touchgene Techne, Cambridge, England). Each cycle had a denaturing step of 95 °C for 1 min, an annealing step

Table 3. Selected *Trichoplusia ni* primers for *rs5* and *apn1* transcripts amplification.

T. ni aminopeptidase 1 (TnAPN1)	Forward: 5' TTGCTGTGAGGAGATTGGCT3' Reverse: 5' CCACTCGATATTGCTCTTAG3'
T. ni ribosomal protein small subunit 5 (S5)	Forward: 5' CGACAGCATGCCTTTACCGC3' Reverse: 5' CAT CCACAGCCTGACGACGC3'

of 55 °C for 1 min, and an extension step of 72 °C for 2 min. Ten microliters of the amplified sample was analyzed in a 1.5% agarose gel and ethidium bromide stain under UV light using a UVP trans-illuminator (VWR, Aurora, Ohio, USA). Optical densities of the DNA bands were quantified using a UVP spectrometer (BioSpectrum Imaging Systems, UVP, Inc., Upland LLC, California, USA). The intensities of the amplified target fragments were quantified using ImageJ 1.42q software Windows version of NIH Image (http://rsb.info.nih.gov/nih-image/). The relative DNA value of each *tnapn1* band was calculated based on the intensity of the corresponding *rs5* band intensity. The relative density was calculated by dividing the *tnapn1* percent value from each sample per treatment by the percent value for the *rs5* (used as endogenous control gene) (Gassmann et al. 2009).

Results

ENZYME ASSAYS

Trypsin and chymotrypsin activity assays performed in triplicate showed consistent results. Relative trypsin and chymotrypsin activities were compared among unexposed laboratory (NL, US), field (GTO), laboratory Xentari-exposed (USX), or field Xentari-exposed (GTOX) strains, using trypsin and chymotrypsin substrates in a microplate assay (Table 4). Chymotrypsin was higher than trypsin activities in laboratory strains (NL and US) with ratios of 1:4.4, and 1:3.2, respectively. When the laboratory strain US was exposed to XenTari (USX), the trypsin:chymotrypsin ratio was reduced to 1:2.3. Both the chymotrypsin and trypsin specific activities were significantly ($P < 0.05$) reduced in USX. The field strain GTO had overall significantly higher trypsin and chymotrypsin specific activity compared to the laboratory strains, with a ratio of 1:1.2. However, the ratio of trypsin:chymotrypsin was increased to 1:1.7 in the XenTari-selected field strain (GTOX) compared to that of the unselected strain.

No differences were observed when comparing the protease activity (as azocasein hydrolysis) of midgut enzymes in *T. ni* larvae in the absence or presence of SDS at a concentration of 0.1%, with the exception of NLX strain, which resulted in significantly reduced enzyme activity after exposure to SDS at 0.1% (Table 5). Nevertheless, azocasein hydrolysis by insect proteases was increased 2.5– to 5.5–fold when buffers contained 1% SDS. In contrast, azocasein hydrolysis by commercial trypsin was significantly reduced ($P < 0.05$) (approximately 2– and 40–fold) when SDS was added to the buffer at 0.1% and 1% SDS, respectively. Similarly, hydrolysis of azocasein by commercial chymotrypsin was significantly reduced (more than 50–fold in 1% SDS).

Table 4. Protease activity in midgut of *Trichoplusia ni* strains exposed or not to *Bacillus thuringiensis.*

	BA*p*NA	SAAPF*p*NA	
T. ni strains[1]	Specific activity (U/mg) ± St error	Specific activity (U/mg) ± St error	Ratio[2]
NL	1.33 ± 0.020 d	5.93 ± 0.041 b	1:4.4
US	6.23 ± 0.077 b	19.7 ± 0.156 a	1:3.2
USX	0.067 ± 0.002 f	1.54 ± 0.036 d	1:2.3
GTO	0.40 ± 0.024 ef	0.50 ± 0.039 e	1:1.2
GTOX5	1.62 ± 0.040 d	2.83 ± 0.087 c	1:1.7

[1]See Table 1 for strains source information. One unit of enzyme activity was defined as the amount which yielded an increase in A_{420} of 0.01 in 30 m at 30 °C (Secades & Guijarro 1999). Different letters after specific activity values indicate differences at $P = 0.05$. Standard error (St error) and ANOVA ($F_{20, 014} = 7742.7$. $P > 0.001$) were calculated using SPSS package version 17.0.

[2]Ratio of specific activity (trypsin:chymotrypsin).

Table 5. Azocasein hydrolysis in midgut extracts from larvae of *Trichoplusia ni* strains in buffers containing increasing amounts of SDS[1].

T. ni strains[2]	Without SDS ± St error	SDS at 0.1% ± St error	SDS at 1.0% ± St error
NL	0.16 ± 0.003 e	0.16 ± 0.003 e	0.61 ± 0.0067 a
NLX	0.18 ± 0.003 d	0.14 ± 0.003 e	0.35 ± 0.003 c
US	0.09 ± 0.003 f	0.09 ± 0.0067 f	0.50 ± 0.0057 b
Trypsin	0.24 ± 0.0067	0.13 ± 0.0057	0.006 ± 0.0005
Chymotrypsin	0.26 ± 0.0057	0.22 ± 0.003	0.005 ± 0.0005

[1]One unit of enzyme activity was defined as the amount which yielded an increase in A_{420} of 0.01 in 30 m at 30 °C (Secades & Guijarro 1999). Different letters after enzyme activity values indicate differences at $P = 0.05$. Standard error (St error) and ANOVA ($F_{14, 1.198} = 1372.8$. $P > 0.001$) were calculated using SPSS package version 17.0.

[2]See Table 1 for strains source information.

ZYMOGRAMS

Zymograms of enzymes from NL, US, GTO and USX strains with casein-infused precast gels (Fig. 1A) or gels incubated postelectrophoresis in buffers containing 1% (Fig. 1A) were obtained. Using casein-infused gels, all protease activity was in the range of 30–200 kDa (Fig. 1B), although the markers in such gels usually cannot migrate accurately. A more accurate picture of caseinolytic activity was obtained by gels incubated with casein postelectorphoretically, and activities were in the range of 10 to 200 kDa. Results demonstrated similar protease profiles among of *T. ni* laboratory strains, with at least 7 caseinolytic activities which we have labeled P1 to P7.

The hydrolysis of Bt proteins by *T. ni* enzymes in laboratory strains NL and US, field strain GTO, and Xentari-selected USX, GTOX3 (selected for 3 generations) and GTOX5 (selected for 5 generations) was analyzed in zymograms using XenTari® protoxins (Fig. 2A) or Cry1Ac protoxin (Fig. 2B) as substrates. In general, the enzyme activities in NL and US that hydrolyzed Xentari proteins were similar to the P1 to P7 caseinolytic enzymes observed in Fig. 1B. The XenTari-selected USX strain lost the lower P1 and P2 activities but gained a higher molecular mass activity of P6, and the profile resembled GTO and NL+GTO (progeny of a

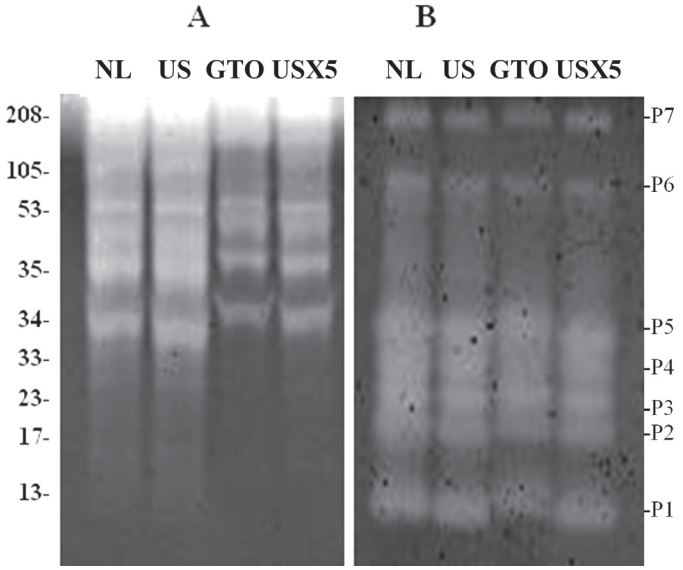

Fig. 1. Zymogram of midgut proteins from *Trichoplusia ni* with casein as substrate. (A) 6-12% Z Blue casein (substrate in gel), or (B) 4-16% Tricine gel, incubated in casein solution postelectrophoresis. Migration of molecular markers is indicated on the left, and proposed *T. ni* protease numbering (P1 – P7) on right, based on migration in the gel.

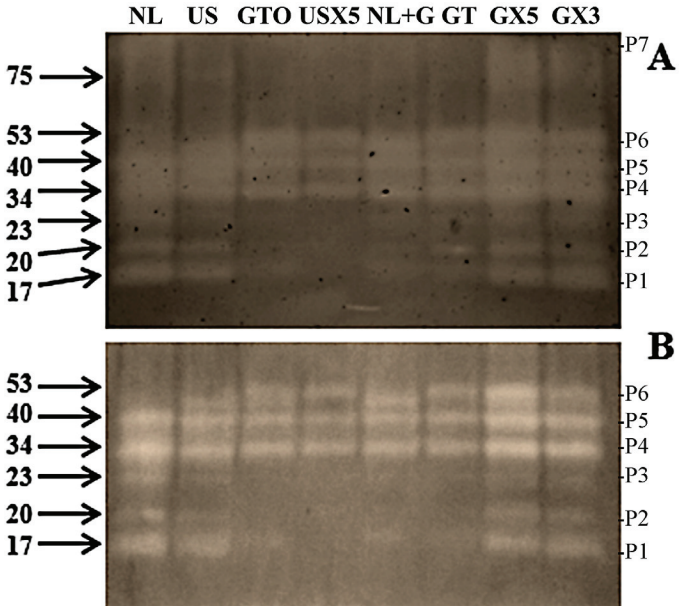

Fig. 2. Zymogram of midgut proteins from *Trichoplusia ni* with either 2% XenTari (A) or 1% Cry1Ac-HD73 (B) as substrate. GT and G represent the GTO strain. Migration of molecular markers is indicated on the left, and proposed *T. ni* protease numbering (P1 – P7) on right, based on migration in the gel.

NL and GTO cross). XenTari selection of GTO (GTOX) resulted in a hybrid profile of all P1 to P7 activities. When a single protoxin was used as substrate, Cry1Ac, the activities were more distinct (Fig. 2B). In this case, only lower molecular mass enzymes were observed, with NL and US exhibiting similar profiles (P1 to P5), and GTO, USX, and NL+GTO with similar profiles (P4 to P6). Selection of GTO with Xentari resulted in the increased expression of the P1 to P3 enzymes observed in NL and US.

ACTIVITY BLOTS

The trypsin and chymotrypsin activities in gut extracts from different *T. ni* strains were compared by substrate specific hydrolysis (Fig. 3). The hydrolysis of BA*p*NA (typical of trypsin-like enzymes), SAAPF*p*NA (typical of chymotrypsin-like enzymes), and SAAPL*p*NA (typical of elastase-like enzymes) was compared in gut extracts from the laboratory strain US, Mexican field strain GTO, and Xentari-selected laboratory strain USX. In US larval extracts, there were 5 trypsin-like activities, P1 and P3 to P6, whereas the GTO extracts lacked the P1 and P5 activities, and the USX extracts lacked the P6 activity (Fig. 3A). Qualitatively, there were at least 3 chymotrypsin-like activities in all 3 enzyme extracts, P1, P2, and P2a, but the activity of extracts from GTO was quantitatively less, even though the same amount of protein was used in the assay (Fig. 3B); there was also a faint higher molecular mass band observed only in the US strain (P4). There were elastase-like activities in all samples (P1, P2, P4, and P7), but overall less activity also was found in GTO extracts (Fig. 3C). Some of the comigration of activities may suggest some enzymes were able to hydrolyze multiple substrates (P1, P2, P4), but alternatively these may be distinct enzymes with similar migration. Substrates P3, P5 and P7 appear to be uniquely trypsin-like enzymes, whereas P7 appears to be uniquely elastase-like.

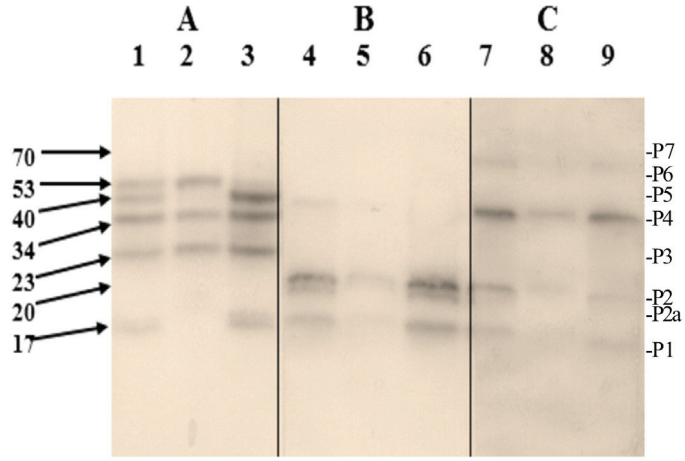

Fig. 3. Detection of protease activity in midgut extracts from different *Trichoplusia ni* strains using class-specific substrates. (A) *N*-a-benzolyl-L-arginine-*p*NA (BA*p*NA) for detection of trypsin-like activity; (B) *N*-succinyl-ala-ala-pro-phe-*p*NA (SAAPF*p*NA) for detection of chymotrypsin-like activity; and (C) *N*-succinyl-ala-ala-pro-leu-*p*NA (SAAPL*p*NA) for detection of elastase-like activity.

TRICHOPLUSIA NI APN1 AMPLIFICATION

We used semi-quantitative RT-PCR to compare the amplification of the *tnapn1* transcript in midguts from selected strains exposed to Xentari to unselected strains reared on normal diet (Fig. 4). Although this assay was done in triplicate, results from 1 gel were used to compare *tnapn1* versus the *rs5* control transcript as amplification reference. Results showed that the amplification of *tnapn1* transcript was 1.9– to 4–fold higher in NLX and USX compared with NL and US, respectively. The amount of amplified *tnapn1* was similar in NL, GTO, and GTOX.

Discussion

Differences in Bt toxin and protoxin susceptibility were previously reported in *T. ni* strains exposed to XenTari (Tamez-Guerra et al. 2006). *Trichoplusia ni* strains from this study included NL that was significantly less susceptible to Cry1Aa protoxin, but was susceptible to Cry1Ab and Cry1Ac protoxin and toxin (Iracheta et al. 2000, Tamez-Guerra et al. 2006). However, while not statistically significant, resistance to Cry1Aa in NL was reduced with the activated form (toxin), suggesting that a protease-mediated resistance may explain the resistance. Midgut enzymes are essential to Bt protoxins solubility, thus their activity is required for toxin efficacy (Segura et al. 2000, Karumbaiah et al. 2007). Alterations in the host midgut protease activities may result in Bt resistance. These findings are similar to previous reports of protease alterations in other insect species that have developed resistance to Bt toxins (Oppert et al. 1997; Candas et al. 2003; Karumbaiah et al. 2007).

In the present study, NL had the lowest trypsin to chymotrypsin activity ratio, which may indicate either lower levels of trypsin or higher levels of chymotrypsin activity in this strain, relative to other strains. In fact, the specific activity towards a trypsin substrate with NL samples was lower than the US laboratory strain, but was also lower with a chymotrypsin substrate (Table 4).

Primary protein-digestion in lepidopteran larvae relies largely on trypsin- and chymotrypsin-like serine protease activities. The functional diversity of digestive enzymes can be correlated to the adaptation of insects to host plants as well as to exposure of insects to naturally occurring antagonistic biomolecules (Srinivasan et al. 2006). Activation of protoxin is mediated by midgut enzymes and is a determinant step in the mechanism of Bt toxin action in the insect midgut (Rukmini et al. 2000). Trypsin-like proteases recognize highly basic residues in a substrate, and these enzymes are critical to Bt protoxin activation to enable midgut binding. Although chymotrypsin-like proteases also have been reported as activators of Bt protoxins, these residues recognize

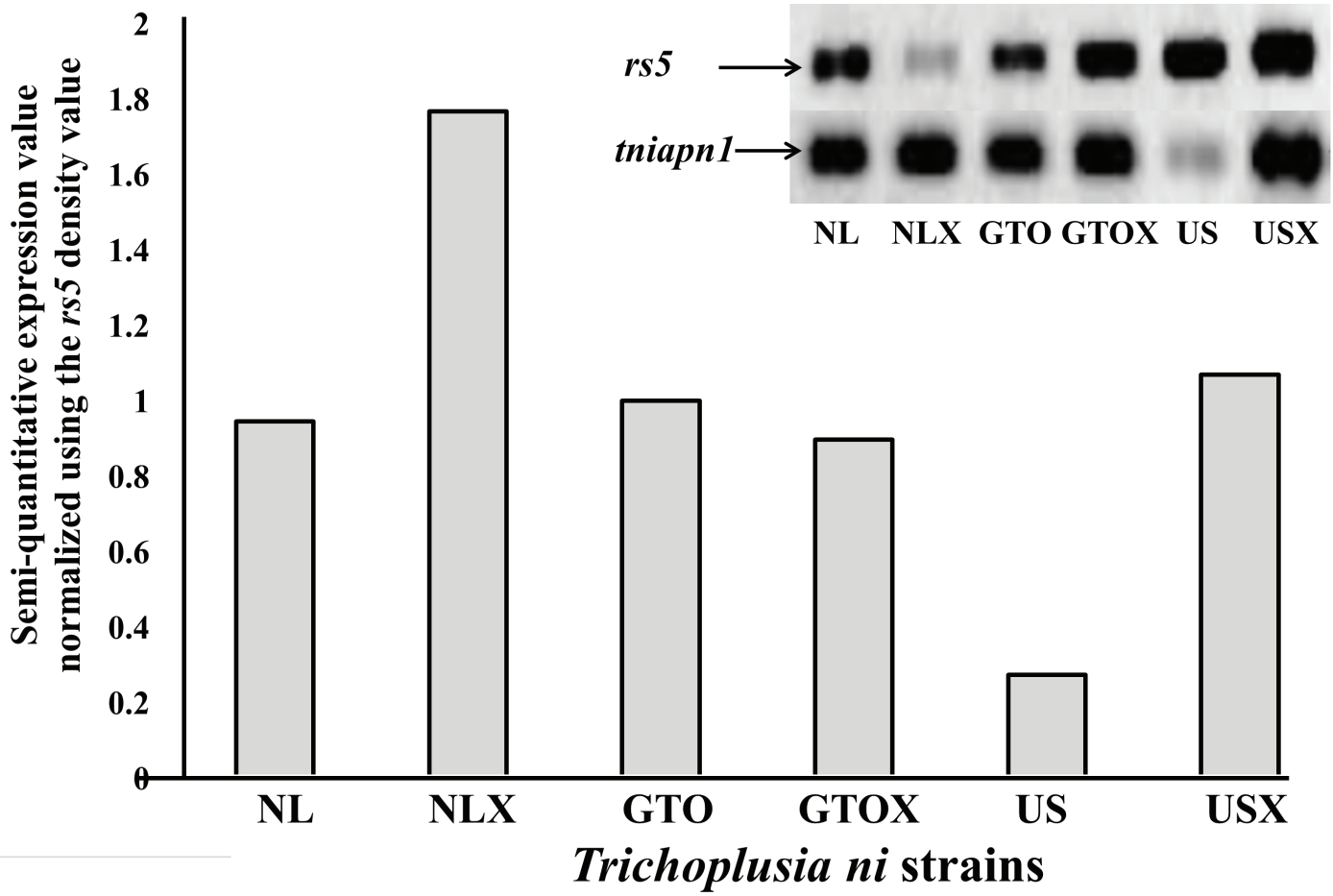

Fig. 4. Relative DNA detected by semi-quantitative RT-PCR using the imageJ software, comparing the *tnapn1* versus the *rs5* control transcript as amplification reference. *Trichoplusia ni* strains NLX, GTOX and USX represent NL, US and GTO after 5 generations being exposed to XenTari.

aromatic residues in a substrate, and it was suggested that chymotrypsin may be more important in the degradation of active toxins (Oppert 1999). Previously, trypsin and chymotrypsin protease activities were reported in *T. ni* midgut fluids at a ratio of about 3:1, respectively, if larvae were collected feeding on plants (Broadway 1989). We used artificial diet and found the opposite, where the trypsin activity was lower compared with that of chymotrypsin, ranging from 1:1.2 to 4.4 in the strains examined in the present study. In fact, chymotrypsin activity was higher among laboratory strains, with trypsin:chymotrypsin ratios of 1:4.4, and 1:3.2 by NL and US, respectively, but there was no clear trend in changes in proteolytic activity in insects selected with Xentari.

SDS has been related to reduction or loss of trypsin hydrolysis capacity (García-Carreño et al. 1993). To assure that SDS would not interfere with our electrophoresis test, we evaluated the effect of this reagent at 0.1 and 1.0%. In contrast to mammalian enzymes, the *T. ni* proteolytic activity was increased with the addition of SDS. After testing the effect of SDS on protease activity of *T. ni* midgut samples, results demonstrated no effect if SDS was used at 0.1% concentration, but azocasein hydrolysis by proteases was increased when 1% SDS was added to the buffer (Table 5). Therefore, *T. ni* enzymes are similarly to other lepidopteran enzymes (Rukmini et al. 2000, Song et al. 2015), being hyperactive at higher SDS concentrations. Since SDS at 0.1% did not affect the enzymatic activity, this concentration was selected for the assays in this study.

We further compared trypsin, chymotrypsin, and elastase activities in *T. ni* larvae, resulting in differential protease profiles among US, GTO, and USX. Overall, activity from GTO was lower than that of US, and USX, although an equal amount of protein was loaded. When the US strain was selected with Xentari (USX), higher molecular mass trypsin and chymotrypsin activities were lost. Five major trypsin activities were observed in US, 4 in USX, and only 3 in GTO (Fig. 3). Three major chymotrypsin activities were observed in US, whereas USX and GTO had only the first 2 activities, but the activities were very low in GTO. The protease activity of trypsin and chymotrypsin in midgut luminal fluids has been documented in many lepidopterans; in contrast, elastase and caseinase activities are scarcely reported. In *Lymantria dispar* L. (Lepidoptera: Erebidae), an elastase-like activity was reported (Valaitis 1995), but this activity was not found in *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) (Coates et al. 2006). In the present study, we found elastase-like activity in all *T. ni* strains tested. Extracts from all *T. ni* strains showed a similar enzymatic activity profile with the elastase substrate (although GTO was again much lower), suggesting a marginal role in the susceptibility to Bt.

Caseinase activity has been reported in several Lepidoptera (García-Carreño et al. 1993, Oppert et al. 1996, Mohan & Gujar 2003). In this study, casein was tested in precast gels (casein incorporated) or incorporated after electrophoresis. Casein incorporated gels revealed that midgut extracts grouped into 2 profile patterns, 1 with enzymes from laboratory strains NL and US, and the other with enzymes from field (GTO) and a laboratory Xentari-exposed strain (USX). When enzymes migrate in a substrate-gel, they can be slowed by interaction with the substrate, and this also can be affected by the amount of enzyme in the sample. However, there were no differences in profile patterns among *T. ni* strains with infusion of casein into the gel fol-

lowing electrophoresis, suggesting that all strains contained 6 major caseinolytic activities.

Oppert et al. (1996) reported differences in Cry1Ac activation with enzymes from Bt resistant strains of *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae). When Bt protoxins were used as substrates in gels, the enzymatic activity profiles of *T. ni* enzymes were different for Cry1Ac or XenTari. The enzymatic activity profile of *T. ni* larvae does not change over generations if they are not exposed to Bt (XenTari, data not shown), but differences were observed after Bt exposure. Protoxin-hydrolyzing enzymes from GTO, USX5, and NL+GTO (strong activities of P4-P6) were substantially different from NL and US (P1-P5), and GTOX3/5 (P1-P6).

The GTO strain was collected from a field where Bt-based commercial products have been applied for 5 to 12 years (Salazar-Solís 2002); this strain was initially field-exposed, and then laboratory-exposed to Bt. GTOX had differences in physiological fitness compared with other *T. ni* strains (reduced larvae weights and lengths, pupa lengths and pupation percentage) (Tamez-Guerra et al. 2006). Because Bt protoxins must be partially digested by proteolytic enzymes to be active, alterations in enzymatic activity can induce fitness costs (Janmaat & Myers 2003, Tamez-Guerra et al. 2006, Janmaat et al. 2014). A previous report with *P. interpunctella* field populations showed same fitness-cost to Bt resistance relationship (Oppert et al. 2000). Activity blots and zymograms showed that the Bt field strain GTO did not have lower molecular mass P1 to P3 activities. The influence of these proteases in the Cry protoxins and toxin hydrolysis, and in insect nutrition and physiological development, is not yet clear.

The mechanisms behind fitness cost among *T. ni* populations resistant to Bt are unknown. Differences in proteolytic activity among the strains may be a result of genetic diversity and may be influenced by the artificial diet (Broadway 1989; Lam et al. 2000). However, since we consistently used the same ingredients and production lot to develop larvae to the 4th instar, it was unlikely that the protease variations were due to the diet. We found that *T. ni* populations collected at the same geographic area (Guanajuato, Mexico) had different susceptibility to Bt (Tamez-Guerra et al. 2006), and several immune response markers were correlated (Tamez-Guerra et al. 2008), thus supporting the genetic diversity hypothesis for the observed differences in *T ni* strains.

Recently, 2 populations of *T. ni* developed resistance to Bt formulations (Bt sprays) in a commercial greenhouse (Kain et al. 2015). When these resistant strains were fed Bt-cotton plants expressing pyramided Cry1Ac and Cry2Ab (BollGard II), about 20% of larvae survived. The first Bt-cotton event available worldwide was Bollgard®, which expresses Cry1Ac toxin. Nevertheless, toxin expression declined with plant age. Bollgard was replaced with Bollgard II® in 2004, which contains Cry2Ab plus Cry1Ac in a pyramid-type event (Olsen et al. 2005). They concluded that selection of insect populations by Bt sprays can lead to cross resistance to different Cry toxins and thus resistance to multitoxin Bt crops. Nevertheless, there are no reports of *T. ni* field populations developing resistance to Bt crops, even though for more than 20 years commercial Bt products have been applied in Mexico for the control of the crucifer pest complex *P. xylostella* and *T. ni.*

A previous study of laboratory and field collected strains of *T. ni* revealed subtle differences in Bt susceptibility (Janmaat et al. 2004). Many reports of *T. ni* strains with resistance to Bt preparations have been associated with Cry1Ab and Cry1Ac resistance in the laboratory and greenhouse, respectively (Janmaat & Myers 2003; Wang et al. 2007; Tiewsiri & Wang 2011; Zhang et al. 2012), where the Cry1Ab/Cry1Ac binding site in the midgut was lost. This loss of binding has been associated with changes or lack of expression of the aminopeptidase N (APN) gene family (Tiewsiri & Wang 2011; Zhang et al. 2012; Park et al. 2014). Reduced binding of Cry toxins to APN has been associated to Cry1A toxin resistance within *T. ni* populations in greenhouse (Wang et al. 2007). More specifically, Cry1Ac resistance has been correlated with lower APN1 and APN6 expression (Tiewsiri & Wang 2011). Using primers for APN1, our results showed higher *tnapn1* transcript amplification in laboratory *T. ni* strains exposed to XenTari. However, no differences in APN1 expression were observed in the field-collected strain GTO and Xentari-selected GTOX. Nevertheless, the susceptibility by GTOX Cry1Aa and Cry1Ab, both protoxin and toxin, was more than 10–fold higher compared with that of Xentari-selected laboratory strains NLX and USX (Table 2). Therefore, higher APN1 expression does not appear to be related to Cry1Aa nor Cry1Ab-resistance in our Xentari-selected *T. ni* strains.

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