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Identification and characterization of digestive serine proteases from inhibitor-resistant *Helicoverpa zea* larval midgut☆

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Abstract

Protease inhibitors mediate a natural form of plant defence against insects, by interfering with the digestive system of the insect. In this paper, affinity chromatography was used to isolate trypsins and chymotrypsins from *Helicoverpa zea* larvae, which had been raised on inhibitor-containing diet. Sensitivity of the fractions to inhibition by plant proteinase inhibitors was tested, and compared to the sensitivity of proteinases found in insects raised on diet to which no inhibitor had been added. The isolated chymotrypsin activity was found to be less sensitive to plant protease inhibitors. The sensitivity of the isolated trypsin activity was found to be intermediate between completely sensitive trypsins and completely insensitive forms that have been previously described. Mass spectrometry was used to identify one trypsin and two chymotrypsins in the partially purified protease fraction. The sequence features of these proteases are discussed in relation to their sensitivity to inhibitors. The results provide insight in the enzymes deployed by *Helicoverpa* larvae to overcome plant defence.

Keywords: Affinity chromatography; Gut; *Helicoverpa zea*; Protease inhibitor; Trypsin

1. Introduction

Insects are a major threat in the lifecycle of a plant, and cause significant economic losses by feeding on crop plants, such as maize, tomato and cotton. Larvae of the lepidopteran insect species *Helicoverpa*, referred to as cotton bollworm (*H. armigera*) or corn earworm (*H. zea*), are a pest in Asia, Australia and the Americas [1]. Many plants react to insect feeding by the synthesis of protease inhibitors [2]. The inhibitors are thought to function by blocking the digestive proteases in the larval gut, thereby interfering with the supply of amino-acids from food protein. As a crop-protection strategy, transfer of genes encoding protease inhibitors has been used to produce resistant transgenic plants. In a number of cases this strategy proved to be successful [3–5], but not for *Helicoverpa* spp., and a few other pest insects. Although plant protease inhibitors appear to block a major part of *Helicoverpa* gut protease activity, and cause growth retardation [6–8], no inhibitors have been identified which prevent *Helicoverpa* larvae from growing to maturity [7,9].

The poor effectiveness of dietary protease inhibitors on this insect probably relates to an adaptation of *Helicoverpa* spp., and a number of other polyphagous insects, to protease inhibitors. The larvae alter the complement of proteolytic activity in their gut in response to inhibitor ingestion, inducing the synthesis of new proteases that are insensitive to the plant inhibitors [10,11]. To elucidate the mechanism behind this phenomenon, a number of trypsin and chymotrypsin cDNA clones have been isolated from *Helicoverpa* mid-guts [8,12], and changes in their expression upon inhibitor ingestion have been probed. However, such data do not provide insight in the abundance of the proteases in the larval midgut, to their importance in protein digestion, and to their sensitivity to inhibitors. In a recent report, we have described the purification and characterization of trypsins directly involved in resistance to plant protease inhibitors from *H. zea* guts [13]. In this paper, the partial purification and char-

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acterization of another trypsin and two chymotrypsins that are induced upon SKTI ingestion are described.

2. Experimental

2.1. Insects

H. zea eggs were purchased from French Agriculture (Lamberton, MN), and hatched at 28 °C on artificial diet as described in [13]. Each individual 1st instar larva was sealed into a chamber containing 5 ml artificial diet. In the final stages of the 4th instar, 50 larvae that were about to molt were transferred to artificial diet supplemented with 0.5% (w/v) (250 μM) SKTI soybean trypsin inhibitor (type II-S, Sigma, St. Louis), while another 50 larvae remained on artificial diet without inhibitor. After 48 h, insects were chilled on ice, and guts with contents were excised, aliquoted and frozen at −80 °C. Frozen guts were thawed on ice, and mixed 1:3 with 50 mM Tris–HCl pH 8 containing 1% polyvinylpolypyrrolidone with 0.5 M NaCl, leading to about 10 ml per 50 guts. Guts were homogenized three times using an S541 potter tube at 60 rpm, and centrifuged for 15 min at 10,000 × *g*, 4 °C to remove solid particles. The supernatant was filtered over a 0.22 μm filter.

2.2. Affinity chromatography

Mustard trypsin inhibitor 2 (MTI-2) was produced in *Pichia pastoris* as described [14]. 15 mg MTI-2 was coupled overnight at 4 °C to 1.5 g CNBr-activated sepharose 4B (Amersham Pharmacia Biotech AB, Upsala) in 20 ml 1 mM HCl according to instructions. The material was used to pour a 5 ml MTI-2 column, which was equilibrated with E-buffer (50 mM Tris pH 8, 0.5 M NaCl). In total 4 ml gut content supernatant of *H. zea* (4 mg total protein) was loaded on the column, after which it was washed with 35 ml E-buffer and 35 ml E-buffer without NaCl. MTI-2-bound protein was eluted stepwise using 5 ml of G-buffer (0.1 M HCl/glycine) pH 3.2; 5 ml G-buffer pH 2.2; 5 ml G-buffer pH 1.5 and 5 ml G-buffer pH 1.5 + 20% DMSO. Eluted fractions were brought to pH 8 using 2 M Tris pH 10.5 and stored at +4 °C.

2.3. Protease assays

Protein fractions were normalized for protein content. In total, 2 μg protein were mixed with 150 μl assay buffer (25 mM glycine/NaOH pH 10; 0.1 mg/ml BSA; 2.5 mM CaCl2). BSA was used to activate proteolytic activity. After 30 min incubation at 22 °C, 50 μl of substrates in assay buffer containing 10% DMSO were added to a final concentration of 1 mM, and substrate breakdown was monitored at 405 nm. Substrates were Z-Arg-Arg-*p*-nitroanilide (Z-RRpNA, Sigma St. Louis) for trypsin, and *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (SAAPLpNA, Bachem Bubensdorf) for chymotrypsin activity. Activity was compared to buffer solutions used to elute proteolytic activity, set at pH 8 by 2 M Tris as described in the previous section. Inhibition by plant protease inhibitors was performed as described in Volpicella et al. [13].

2.4. SDS–PAGE and iso-electric focusing

For SDS–PAGE, 2.5 μg of total gut proteins and 0.5 μg (15 μl) of fractions from affinity chromatography showing proteolytic activity were diluted with 5 μl of sample buffer (20% glycerol, 20 mM Tris pH 6.8, 0.4% sodium dodecyl sulfate, 0.001% Bromo phenol blue), and kept on ice. Protein staining was done using silver nitrate according to Rabilloud et al. [14]. Staining for general proteolytic activity with casein was done according to Jongsma et al. [15].

For *iso*-electric focusing (IEF), 300 μl of fractions (10 μg protein) were precipitated with 10% TCA, washed with ice-cold acetone and resuspended in 125 μl rehydration solution (8 M urea, 2 M thio-urea, 2% CHAPS, 2 mM dithiothreitol and 2 mM EDTA). 0.5% IPG buffer pH 6–11 (Amersham, Upsala) was added, mixed and the sample was allowed to enter an Immobiline DryStrip pH 6–11 (7 cm; Amersham) overnight. The focusing was performed for 6 h from 500 to 8000 V. The strip was subsequently equilibrated in a solution containing 1% DTT, 50 mM Tris–HCl pH 8.8, 6 M Urea, 30% glycerol and 2% SDS, and stained with Coomassie Brilliant Blue.

2.5. Mass spectrometry and sequence matching

Protein bands from gel were excised, dried, and digested in gel with trypsin, according to [16]. Proteins were extracted, and loaded onto a C18 PepMap column (15 cm × 75 μm). Peptides were eluted by a 30 min. gradient from 0.5% formic acid in water to 0.5% formic acid in 50% acetonitril at a speed of 0.2 μl/min. The C18 column was connected to the electro-electro-spray of a Q-TOF-2 Mass Spectrometer (Micromass) by a PicoTip (New Objective). The QTOFmass spectrometer was instructed to determine charge of the eluting peptides, and, if appropriate (i.e. 2+ or 3+), the QTOF-MS switched to the MS/MS mode applying collision-induced dissociation (CID). The resulting CID spectrum contains the sequence information for a single peptide.

The ProteinLynx GlobalServer package V2.1 (Waters) was used to process MS/MS data. Raw MS/MS spectra were deconvoluted to produce mono-isotopic singly charged spectra with the proprietary MaxEnt3 algorithm. MS/MS spectra containing good quality CID products were automatically selected for BLAST analysis against known peptides from the NCBI nr database. Unassigned MS/MS spectra were automatically processed using the AutoMod algorithm, developed to identify amino acid substitutions, post-translational modifications and partial or non-specific cleavages. De novo sequences were generated with the MassSeq tool to search for the most likely protein homologs in the databanks. The MS/MS spectra (around 25 per peptide) were further scrutinized by hand using the ManSeq mode.

3. Results and discussion

3.1. Enzyme purification and activity

H. zea larvae were reared on two different diets, one containing SKTI, and the other one without added inhibitor. Gut

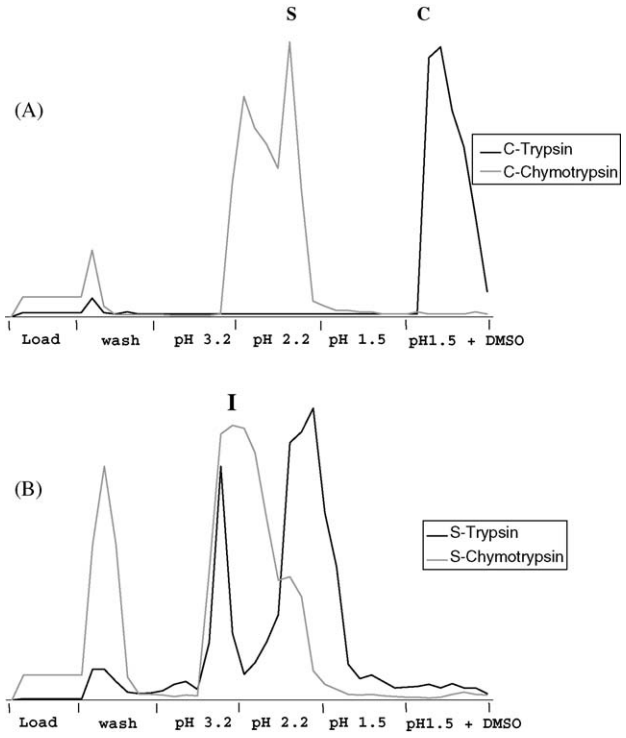

Fig. 1. Activity profile of fractions eluted from the affinity column of control-diet gut content (A) and SKTI-diet gut content (B). Elution steps are indicated by the pH value of the used buffer reported along the *X*-axis. *Y*-axis indicates relative proteolytic activity, expressed as increase in absorption at 405 nm/min, representing substrate hydrolysis. Washing step is not in scale. Substrates were Z-RRpNAfor trypsin and SAAPLpNA for chymotrypsin. I, S and C refer to fractions HzTrypsin-I, HzTrypsin-S and HzTrypsin-C.

content of the larvae was isolated and crude extracts were used for purification of trypsin and chymotrypsin enzymes by affinity chromatography with CNBr-Sepharose immobilized MTI-2.

Fig. 1 shows the proteolytic activity of fractions eluted from crude extracts of *H. zea* larvae reared on control diet (A) and SKTI diet (B). Elution was performed by stepwise lowering of the pH. Proteolytic activities were detected by using trypsin and chymotrypsin-specific substrates (Z-RRpNA and SAAPLpNA respectively). The MTI-2 column concentrated trypsin activity of the control diet in a single peak, eluting at pH 1.5 with 20% DMSO. In contrast, trypsin activity of the SKTI-diet larvae eluted at pH 3.2 and 2.2. Also chymotrypsin activity differed in the elution profiles of both diets (Fig. 1). Two peaks were observed in the control diet (pH 3.2 and 2.2). In the SKTI diet gut extract, a major broad peak appeared at pH 3.2, and a large portion of chymotrypsin activity was not retained by the column.

The control diet trypsin eluting at pH 1.5 with 20% DMSO (HzTrypsin-C; C in Fig. 1) and the SKTI diet trypsin eluted at pH 2.2 (HzTrypsin-S; S in Fig. 1) have already been extensively characterized [13]. In this paper, we focus on trypsins and chymotrypsins in the SKTI-diet larvae, eluting at pH 3.2 from the affinity column. In gut extracts from both diets, this fraction contained chymotrypsin activity, while in the SKTI diet gut extracts also a trypsin activity was detected, which was not present in the control diet guts. Apparently this fraction contains an SKTI-induced trypsin (HzTrypsin-I; I in Fig. 1).

Even if MTI-2 can be considered as a weak inhibitor of chymotrypsins [17], it appeared to be effective for the isolation of chymotrypsins by affinity chromatography, and appropriate for the identification of adaptive chymotrypsins. Indeed, analysis of proteolytic activity eluted in the HzTrypsin-I fraction showed the presence of chymotrypsins.

3.2. Protease sensitivity to PIs

The effect of plant protease inhibitors on the activity of semi-purified trypsins and chymotrypsins was tested. Midgut extracts of *H. zea* larvae reared on control and SKTI diets were examined for sensitivity to four inhibitors belonging to different families: potato inhibitor II (PI-2), a double headed inhibitor inhibiting both trypsin and chymotrypsin, representative of the potato inhibitor II family, only found in *Solanaceae* [18]; mustard trypsin inhibitor II (MTI-2), which is an inhibitor of trypsin but not chymotrypsin, and belongs to the MSI family, only found in *Cruciferae* [17]; soybean Bowman-Birk inhibitor (SBTI), a double-headed trypsin and chymotrypsin inhibitor belonging to the Bowman-Birk family primarily present in *Leguminosae* [19]; soybean Kunitz trypsin inhibitor (SKTI), a trypsin inhibitor of the Kunitz family [20], which has representatives in most plant species. SKTI has a single reactive site, specific for trypsin, but it has been reported that insect chymotrypsins can also be strongly inhibited [21].

Chymotrypsin activity (probed as activity on SAAPLpNA; Fig. 2A) of the total midgut extract was clearly affected by SKTI ingestion. When inhibitors were tested at 0.4 μM concentration, chymotrypsin activity of the control total gut extract was inhib-

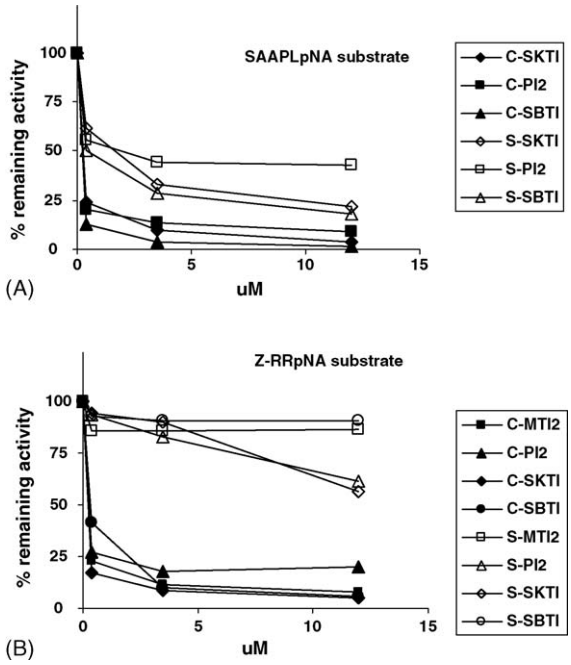

Fig. 2. Inhibition assays on proteolytic activity of total gut extracts. Assays were carried out using the trypsin-specific substrate (Z-RRpNA) and the chymotrypsin-specific substrate (SAAPLpNA) in the presence of different PIs (see legends inside each box). Extracts were from guts of *H. zea* reared on control (solid symbol) and SKTI containing (open symbols) diets.

ited by 76% with SKTI, 80% by PI-2 and by 87% with SBTI. Chymotrypsin activity in midgut extracts from SKTI-fed larvae was only partially inhibited by plant PIs: 40% by SKTI, 44% by PI-2 and 50% by SBTI (Fig. 2A). MTI-2 had no effect on chymotrypsin activity on both diets (not shown). Thus, a significant portion of the chymotrypsin activity present in guts from SKTI fed larvae had become insensitive to inhibition by chymotrypsin inhibitors.

Midgut trypsin activity, as reported before [21], was affected more drastically by the presence of SKTI in the diet. Activity (probed as activity on Z-RRpNA) in control-diet gut extract was inhibited by 83% with SKTI, by 72% with PI-2, by 60% with SBTI, and by 77% with MTI-2 (Fig. 2B). When midgut extract from SKTI-diet was tested, hardly any inhibition could be observed (5% by SKTI, 6% by PI-2, 7% by SBTI, and 14% by MTI-2 (Fig. 2B)). Even if the SKTI has no effect when tested on bovine chymotrypsin activity, it appears to have a significant effect on chymotrypsin activity in the gut of *H. zea* larvae [21]. While the effect of SKTI ingestion on trypsins is strong, with a reduction of sensitivity to SKTI of up to 83%, the effect on chymotrypsins is rather mild, reducing their activity by up to 75% (Fig. 2).

This effect of SKTI ingestion on chymotrypsins is substantiated by observations made during and after the affinity purification. The MTI-2 affinity column retains most chymotrypsin activity from the control diet gut extracts; in contrast, a large portion of chymotrypsin activity in the SKTI diet gut extracts appears to have no affinity for the column, and is found back in the flow through (Fig. 1).

After purification, inhibition assays on single affinity fractions were also performed (Fig. 3). Chymotrypsin activity in fractions eluting at pH 3.2 was assayed, both for control and SKTI gut extracts (Fig. 3A). The two fractions showed a different sensitivity to the tested plant PIs. At 0.4 μM inhibitor concentration, the control chymotrypsin fraction was inhibited by 45% with PI-2, by 37% with SBTI, and only by 10% with SKTI. In contrast, the SKTI chymotrypsin fraction was not inhibited at 0.4 μM concentration, but only at higher concentration.

The control trypsin fraction (HzTrypsin-C) is completely inhibited by the four different plant PIs tested (Fig. 3B_1), while SKTI-trypsin eluting at pH 2.2 (HzTrypsin-S) shows an almost complete insensitivity (Fig. 3B_2) [13]. The HzTrypsin-I fraction, eluting at pH 3.2, appears to be more sensitive to inhibition than the previously characterized HzTrypsin-S (Fig. 3B_3). It is however not as sensitive to inhibitors as HzTrypsin-C, and trypsin concentration in the HzTrypsin-I fraction is probably lower (about five-fold, as judged by RRpNAse activity; not shown) than in the HzTrypsin-S and HzTrypsin-C fraction. The lower concentration of HzTrypsin-I could explain why less inhibitor is required to quench it, in comparison to HzTrypsin-S. Therefore, we consider HzTrypsin-I as a trypsin that is insensitive to inhibition, though maybe not to the same extent as HzTrypsin-S.

3.3. Identity assignment of enzymes by mass spectrometry

In order to assess which chymotrypsins and trypsins were present in the pH 3.2 fraction, further analyses by proteomics approaches were performed. Firstly proteins of the pH 3.2 fractions of the SKTI-fed insects and control-fed insects were separated on IEF strips covering a pH range of 6–11 (Fig. 4). No bands were observed on the IEF strip for the control-fed insects. IEF of the pH 3.2 fractions of the SKTI-fed insects showed a single dominant band (M7) that migrated around pI 9–10, and five minor bands.

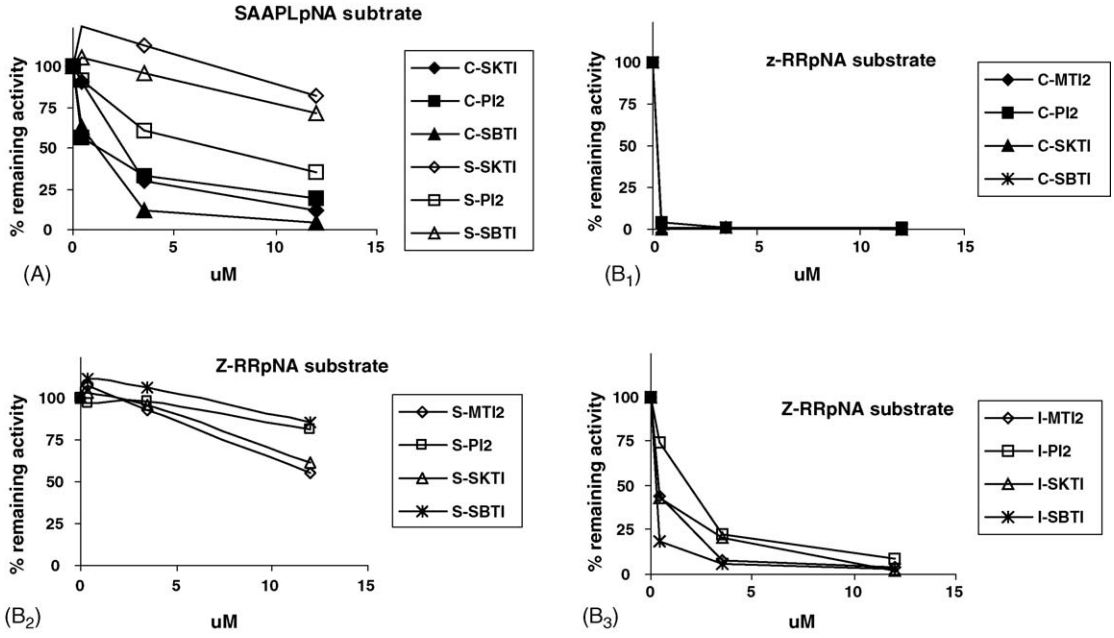

Fig. 3. Inhibition assays on single affinity fractions. Proteolytic activity was assayed using the trypsin-specific substrate (Z-RRpNA) and the chymotrypsin-specific substrate (SAAPLpNA) in the presence of different plant PIs (see legend in each box). (A) Refers to fractions from control and SKTI diets eluted at pH 3.2. (B_1–B_3) Refer to fractions obtained from control diet at pH 1.5 + 20% DMSO, and from SKTI diet at pH 2.2 and 3.2, respectively. For all the figures, solid symbols refer to control diet, open symbols to SKTI diet.

Fig. 4. Iso-electric focusing of pH 1.5 + 20% DMSO fraction from control diet and pH 3.2 fraction from SKTI-diet. Ten micrograms of total proteins were loaded on a pH 6–11 strip and electrophoresed.

The M7 band was further analysed by MS. Proteins were eluted from the gel, digested by bovine trypsin and analysed by means of a Q-TOF-2 Mass spectrometer (Micromass). A more accurate assignment to trypsin cDNA sequences present in the databases was achieved by MS/MS sequencing of selected trypsin fragments. Peptides found in the M7 band corresponding to proteases are listed in Table 1. Poorly matching peptides, or peptides matching to other database accessions were in general found to be low-abundant peptides, while all major peptides matched protease sequences.

Several peptides indicate the presence of a polypeptide corresponding to a cDNA with accession HaY12275. HaY12275 encodes a gut-expressed trypsin from *H. armigera*. Five peptides correspond exactly to HaY12275, while two more peptides require a substitution before they match. Substitutions may arise from the fact that the HaY12275 sequence derives from *H. armigera*, while the sequenced peptides derive from *H. zea*. Overall, 130 out of 233 residues (56%) of the mature protein encoded by Ha12275 are covered by the peptides identified by the MS analysis.

Peptides corresponding to chymotrypsin cDNAs in the database were also identified. Two populations of peptides match to chymotrypsins: one set of six peptides indicates the presence of a protein corresponding to accession HaY12279. These peptides cover 99 out of 234 amino-acid residues (42%) encoded by the HaY12279 cDNA. Another set of five peptides correspond to accession HaY12287. Again, in the case of HaY12287, three minor amino-acid changes were detected in the peptides, relative to the database cDNA. The peptides cover 30% (70 out of 234 residues) of the mature protein encoded by HaY12287.

Previously published expression analyses confirm that the chymotrypsins we identified indeed are upregulated upon SKTI ingestion. By Northern blotting, the mRNA HaY12279 was found to be induced in *H. armigera* guts after feeding on SKTI-diet [8], which is in agreement with our observation that this enzyme occurs as a reaction to SKTI ingestion. Peptides matching to cDNA HaY12287 also match to a number of closely related cDNAs (accessions HaY12280, HaY12281, HaY12284, HaY12285, AF233734). The homology between these isoforms is very high (>95% identity at the DNA level), for which reason it is difficult to determine exactly which of these isoforms are represented in the gut extract. Northern hybridisation of the HaY12284 sequence, which is highly homologous (97% identi-

Table 1
Peptides identified in the pH 3.2 fraction of the gut extract from SKTI-fed larvae

Database match[a]	Molecular weight[b]	Start[c]	Sequence[d]	Substitutions[e]
HaY12287	988.457	198	(R)DGGSVDGNLR(H)	
HaY12287	1162.555	272	(R)GCQVGSPAAFAR(V)	
HaY12287	1205.702	260	(R)ILIGVTSFGTAK(G)	T for S (10), K for R (12)
HaY12287	1822.811	241	(R)STCQGDSGGPLVVTSNNR(R)	
HaY12287	1968.036	133	(R)LNTASVVMHGSFNPNLIR(N)	F for W (12)
HaY12279	1146.56	253	(R)GCQVGAPAAFAR(V)	
HaY12279	1263.682	241	(R)ILIGVTSFGSDR(G)	
HaY12279	1365.704	265	(R)VTSYISWINQR(L)	
HaY12279	1822.811	222	(R)STCQGDSGGPLVVTSNNR(R)	
HaY12279	2038.005	114	(R)LNSNSVVMHGSWNPNLIR(N)	
HaY12279	2820.419	149	(N)IAPIALPSGNELNNQFAGFTATASGFGR(T)	
HaY12275	1130.629	21	(R)IVGGTVTTIDR(Y)	
HaY12275	1141.645	108	(R)TLNNDIAILR(S)	
HaY12275	1449.715	60	(R)ALLTAAHCTHNRGA(V)	K for R (13)
HaY12275	1679.878	167	(R)HVQLVIINQNTCKN(N)	R for Q (23)
HaY12275	2932.469	81	(R)VGSTWANSGGVVHNVNVNIIHPRFDSR(T)	
HaY12275	3230.687	32	(R)YPTIAAMLYAPNGITFGQSCGGTILNNR(A)	
HaY12275	3768.741	204	(R)DQCQGDSGGPLYHNGIVVGVSSFGIGCGNAFFPGVSAR(V)	

[a] The accession number of the most representative cDNA sequence in which this peptide has been identified.
[b] Calculated mass of the peptide.
[c] Position of the first amino acid of the peptide in the protein encoded by the cDNA.
[d] Amino acids between brackets represent residues that are not part of the peptide, but neighbour it in the cDNA sequence.
[e] Potential substitutions of the peptides relative to the cDNA sequence.

cal DNA residues) to HaY12287, indicates strong up-regulation upon SKTI ingestion [8].

Our analysis clearly indicates that the cDNAs HaY12279 and HaY12287 (and/or closely related specimens) encode proteins that are found in significant quantities in the midgut upon SKTI ingestion. Although with the present data we cannot discriminate between the two cDNA groups, the mixture of these two is partially resistant to SKTI, SBTI and PI-2, and contributes to protein digestion.

3.4. Sequence comparison of trypsins with different sensitivity

In a previous paper we have reported about the properties of the dominant trypsins in the midgut of SKTI-fed and control-diet-fed *H. zea* larvae [13]. The analysis of protease activities in the pH 3.2 fraction revealed the presence of another trypsin in the SKTI diet gut extract, HzTrypsin-I, which is absent from the control gut extract. The peptide analysis of this fraction indeed confirmed the presence of a trypsin, corresponding to cDNA HaY12275, which likely corresponds to HzTrypsin-I. The expression of the HaY12275 cDNA has been detected in the guts of *Helicoverpa* larvae after feeding on a SKTI-diet [8]. The regulation of HaY12275 expression upon inhibitor ingestion has been recently confirmed by Chougule et al. [22]. *Helicoverpa* larvae fed on host plants were not found to express the HaY12275 mRNA, where larvae did express this mRNA when they were raised on non-host plants, which potentially express PIs effective against *H. armigera* proteases.

It is interesting to study sequence similarities (Fig. 5) between HaY12275, and the trypsins that we have previously identified (HaY12269, from control diet and fully sensitive to inhibition, and HzT15, from SKTI diet, not sensitive to inhibition). HaY12275 is more related to the insensitive HzT15 (85% similarity) than to the sensitive HaY12269. Among the three proteases, five regions can be identified in which there are higher differences in amino acid content (Fig. 5, asterisks). These regions are generally part of exposed loops. The main amino-acid differences between sensitive HaY12269 and resistant HzT15 trypsins have been already described and mapped on the porcine trypsin three-dimensional structure [13]. When compared to 3D structures of serine proteases in complex with inhibitors, the putative contacts of the exposed loops with inhibitors can be anticipated. Some of the exposed loop residues (41, 60, 96, 97, 175, 217) are probably in contact with inhibitors like SKTI, PI-2 and SBTI, while the region around residue 145 is in contact with

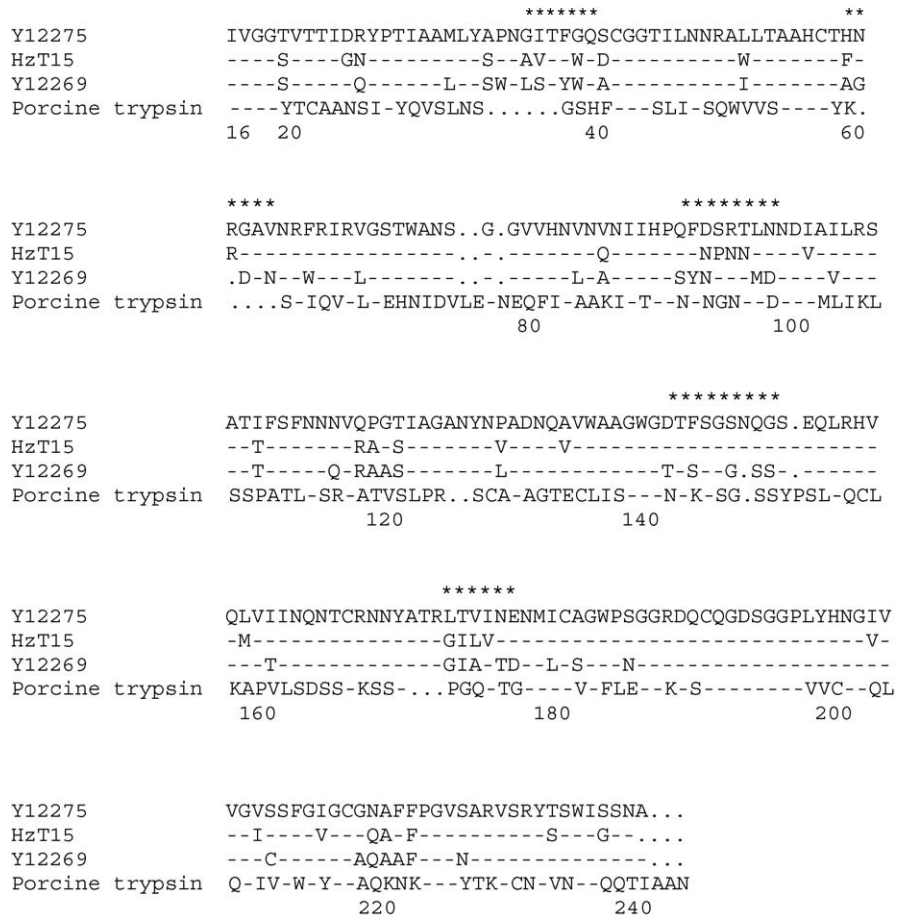

Fig. 5. Alignment of HaY12275 (HzTrypsin-I), HaY12269 (HzTrypsin-C) and HzT15 (HzTrypsin-S) deduced amino acid sequence. Dashed indicate conserved residues. Regions indicated with asterisks contain high amino acid diversity. Porcine trypsin is shown for comparison. Dots have inserted to maximize homology. The numbering is according to the bovine chymotrypsinogen sequence.

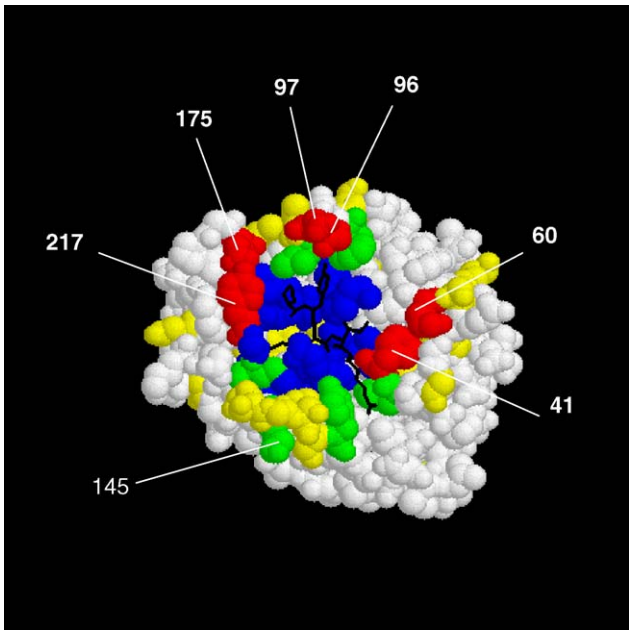

Fig. 6. Space filling representation of the trypsin component of the trypsin-SKTI complex (PDB accession 1AVW) as generated by Rasmol. In black sticks the P3–P2′ residues of SKTI are represented, as analogues of a substrate. Yellow residues are at positions in the alignment where HzTrypsin-C and HzTrypsin-S are different. Blue residues are contacting SKTI. Green residues are the overlap between yellow and blue residues. Red residues are those, among the SKTI-contacting ones, which differ either in the three proteases or only between HzTrypsin-I and HzTrypsin-S. Residues in contact with the SKTI inhibitor are indicated by numbers.

SKTI, but not with PI-2 or SBTI (Fig. 6) [20,19,23]. Residues which contact SKTI and which either differ in the three proteases (41, 60, 175) or are conserved only between HzTrypsin-C and HzTrypsin-I (96, 97, 217) are reported in red in Fig. 6. These residues may be responsible for the different level of sensitivity. Residue 60, for instance, is an Ala residue in Y12269, while in the resistant HzT15 and Y12275 it is a more bulky Phe and His, respectively. There is also a remarkable correspondence between HzT15 and HaY12275, as opposed to HaY12269 and bovine trypsin in the region from residue 140 to 148. The identity of these residues is completely conserved between the SKTI-induced enzymes HzT15 and Y12275, while both in the sensitive enzyme HaY12269, and in bovine trypsin, six out of nine residues either have a completely different identity, or are absent. This suggests that this region is involved in resistance to inhibition.

4. Conclusions

An affinity chromatography system for purification of insect proteases has been deployed. By this approach trypsins and chymotrypsins have been purified that appear to be insensitive to plant PIs. In the larvae of *H. zea*, these proteases obviously play a role in the adaptation of the insect to plant defence. Sequences of isolated proteases were determined by MS analysis. Some residues putatively involved in the insensitivity to inhibitors were identified by structural comparison of the insect proteases. Proteases identified in this study would be good candidates for further studies to interactions with plant PIs, to understand the structural reasons of protease insensitivity.

Acknowledgements

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