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Molecular cloning of trypsin-like cDNAs and comparison of proteinase activities in the salivary glands and gut of the tarnished plant bug *Lygus lineolaris* (Heteroptera: Miridae)

Yu Cheng Zhu ^{a,*}, Fanrong Zeng ^b, Brenda Oppert ^c

^a USDA-ARS-JWDSRC, PO Box 346, Stoneville, MS 38776, USA

^b USDA-ARS, PO Box 5367, Mississippi State, MS 39762, USA

^c USDA-ARS, GMPRC, 1515 College Avenue, Manhattan, KS 66502, USA

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Abstract

Using specific proteinase inhibitors, we demonstrated that serine proteinases in the tarnished plant bug, *Lygus lineolaris*, are major proteinases in both salivary glands and gut tissues. Gut proteinases were less sensitive to inhibition than proteinases from the salivary glands. Up to 80% azocaseinase and 90% of BApNase activities in the salivary glands were inhibited by aprotinin, benzamidine, and PMSF, whereas only 46% azocaseinase and 60% BApNase activities in the gut were suppressed by benzamidine, leupeptin, and TLCK. The pH optima for azocaseinase activity in salivary glands ranged from 6.2 to 10.6, whereas the pH optima for gut proteinases was acidic for general and alkaline for tryptic proteinases. Zymogram analysis demonstrated that ~26-kDa proteinases from salivary glands were active against both gelatin and casein substrates. Three trypsin-like cDNAs, LISgP2–4, and one trypsin-like cDNA, LIGtP1, were cloned from salivary glands and gut, respectively. Putative trypsin precursors from all cloned cDNAs contained a signal peptide, activation peptide, and conserved N-termini (IVGG). Other structural features included His, Asp, and Ser residues for the catalytic amino acid triad of serine proteinase active sites, residues for the binding pocket, and four pairs of cysteine residues for disulfide bridges. Deduced trypsin-like proteins from LISgP2, LISgP3, and LIGtP1 cDNAs shared 98–99% sequence identity with a previously reported trypsin-like precursor, whereas the trypsin-like protein of LISgP4 shared only 44% sequence identity with all other trypsin-like proteins, indicating multi-trypsin forms are present in *L. lineolaris*.

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1. Introduction

Like many other heteropteran insects, the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), ingests

plant liquids by inserting sucking mouthparts into plant tissues. Extra-oral digestion is facilitated by the secretion of digestive enzymes from the salivary glands (Cohen, 1998). Proteolytic activity has been detected in salivary glands from many mirids, including *Lygus rugulipennis* (Laurema et al., 1985) and *Creontiades dilutus* (Colebatch et al., 2001). In these species, serine proteinases are most abundant in the salivary glands (Goodchild, 1952; Hori, 1970; Cohen, 1993). In the salivary glands of *L. lineolaris*, trypsins are probably major proteinases, as determined by inhibition analysis and molecular cloning (Zeng et al., 2002a).

Currently, control of *L. lineolaris* on cotton is almost exclusively by chemical insecticides. The economic importance of *L. lineolaris* has become prominent due to reduced chemical applications in areas where transgenic

* Corresponding author. Tel.: +1-662-686-5360; fax: +1-662-686-5421.

Abbreviations: BApNA, α -benzoyl-DL-arginine-*p*-nitroanilide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; PCPI, potato tuber carboxypeptidase inhibitor; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; RACE, rapid amplification of cDNA end; RT, reverse transcription; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor; TCA, trichloro acetic acid; TLCK, *N*- α -tosyl-L-lysine chloromethyl ketone; TPCK, *N*- α -tosyl-L-phenylalanine chloromethyl ketone

E-mail address: yczhu@ars.usda.gov (Y.C. Zhu).

cotton with Bt (*Bacillus thuringiensis*) genes is widely used to control lepidopteran insects. Increased pest status, coupled with the increasing prevalence of resistance to pyrethroids and organophosphates (Snodgrass, 1996; Snodgrass and Scott, 2000), has prompted consideration of alternative controls for plant bug on cotton. Bioactive proteins, such as digestive proteinase inhibitors, have potential to be used for insect control (Duan et al., 1996). Genes producing bioactive proteins can be used to transform host plants to suppress insect growth and reduce pest insect population size (Baker and Kramer, 1996), which can subsequently reduce the need for chemical applications. To design and incorporate an inhibitor gene into a host plant genome, knowledge of the biochemistry of protein digestion is necessary, especially the characteristics of enzyme/inhibitor interactions, multi-gene phenomena of proteinases with differential sensitivity to inhibitors, and potential for adaptation to the presence of dietary inhibitors by the production of endoproteinases insensitive to a given inhibitor (Broadway, 1995; Jongsma et al., 1995; Bown et al., 1997).

Zeng et al. (2002a) demonstrated that tryptic activity was present in the salivary glands of *L. lineolaris*, which was suppressible with serine proteinase inhibitors. This study was conducted to determine if *L. lineolaris* has multiple proteinase genes with different expression in salivary glands and guts, to understand if salivary glands and guts play an equal role in protein digestion, and to determine how different proteinase inhibitors influence protein digestion in the salivary glands and guts.

2. Materials and methods

2.1. Insect cultures

Lygus lineolaris adults were collected from weedy areas near cotton fields in Washington County, MS. Some insects were immediately dissected, whereas the remainder were maintained on young green bean seed pods, *Phaseolus vulgaris* L., at 27 ± 1 °C, RH $60 \pm 5\%$ with a 14 h light/10 h dark cycle in a growth chamber until dissection.

2.2. Preparation of salivary gland and gut homogenates

Salivary gland complex (SGC) and midgut were dissected under a stereo light microscope (Nikon SMZ1000) in a cold saline solution (128 mM NaCl, 4.7 mM KCl, 2.8 mM CaCl₂). The SGC (including all lobes, accessory glands and tubules) was exposed by holding and pressing the abdomen, and simultaneously removing the prothorax with forceps. Clear SGC was separated from the head-prothorax with fine forceps. The midgut

was exposed by holding the thorax with one forceps and pulling the terminal abdomen segments with another forceps. Salivary glands or guts were transferred to microcentrifuge tubes and held on ice. After dissection, the SGC and guts were stored at -80 °C until needed. Enzyme extracts were prepared using a method similar to that described by Oppert et al. (1994). For proteinase assays, three pairs of salivary glands or three guts were immersed in 5 μ l of 1 mM dithiothreitol (DTT). Ten pipetting actions were applied in order to break up the tissue. The mixture was then centrifuged for 2 min at 15,000 g at 4 °C. The supernatant was used immediately for enzyme assays.

2.3. pH optimum

Azocasein was used as a general substrate to determine the pH optima of the salivary gland and gut proteinases. BApNA was used as a trypsin substrate to measure the pH optimum of the trypsin-like proteinases in the gut, using the universal buffer system (pH 2.0–11.5) of Frugoni (1957). Five microliter of enzyme extract (equivalent to 3 pairs of salivary glands or 3 guts) was mixed with 50 μ l of universal buffer at each pH (Frugoni, 1957) in a microtiter plate (in triplicate). BApNA solution was prepared by diluting the stock (50 mg/ml in DMSO) with each pH buffer to obtain a final BApNA concentration of 0.5 mg/ml. The BApNAase activity was spectrophotometrically determined after the addition of 45 μ l of diluted BApNA solution. Absorbance at 405 nm was measured every 15 min at 37 °C on a ELx808iu microtiter plate reader (Bio-Tek Instruments Inc., Winooski, VT). Kinetic curves were generated using the KC4 program (Ver. 2.7, Bio-Tek), and the mean V (velocity) was calculated as the increase in milliabsorbance over 6 h (the time when the increase in absorbance was linear) and was used as parameter of proteinase activity. A no-enzyme control was included at each pH to correct for endogenous hydrolysis of substrate. Means of enzyme activity at each pH were calculated from triplicate readings. The experiment was repeated with different enzyme preparations.

Procedures of Walker et al. (1998) with slight modifications were used to measure general proteinase activity using azocasein as substrate. Five microliters of the enzyme extract was mixed with 10 μ l azocasein solution (1% w/v in 0.05% SDS), 20 μ l universal buffer at each pH, and 5 μ l H₂O in a microcentrifuge tube. The tube was incubated for 19 h at 25 °C. After incubation, undigested substrate was precipitated with the addition of 30 μ l 10% TCA. The tube was placed on ice for 30 min and centrifuged at 14,000 $\times g$ for 5 min to remove precipitated protein. Supernatant (60 μ l) was transferred to a well of microtiter plate containing 40 μ l 1 M NaOH. Absorbance was measured at 405 nm.

2.4. Inhibition of salivary gland and gut proteinases

Inhibition studies were conducted to measure BApNAse and azocaseinase activity changes in the presence of different inhibitors. Eleven inhibitors were tested with enzyme extracts from both SGC and gut. Pepstatin A, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), aprotinin, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (STI, Bowman-Birk), chymostatin, *N*- α -tosyl-L-lysine (TLCK), leupeptin, trans-epoxysuccinyl-L-leucylamido-(4-guanido)-butane, (E-64), benzamidine, potato tuber carboxypeptidase inhibitor (PCPI) were obtained from Sigma Chemical Company (St. Louis, MO). Stock solutions were prepared in ethanol for pepstatin A, TPCK, and PMSF, and in dH₂O for the remaining inhibitors. The concentration and target proteinases listed in Table 1 for each inhibitor were obtained from Girard et al. (1998), Ortego et al. (1996), and Elden (1995).

Inhibitors (1 μ l) were preincubated with 5 μ l enzyme extract (equivalent to 3 pairs of salivary glands or 3 guts) and 45 μ l of pH 8.5 universal buffer (Frugoni, 1957) for 15 min at 30 °C in a microtiter plate (in triplicate). Final inhibitor concentrations and corresponding target enzymes of each inhibitor are given in Table 1. BApNAse activity was measured immediately after the addition of 50 μ l of BApNA solution (BApNA stock was diluted with 100 \times universal buffer, pH 8.5, to obtain a final concentration of 0.5 mg/ml). Absorbance was measured at 405 nm, and the kinetic curves for the mean velocity (Mean *V*) were generated using the method described above. The experiment was repeated with different enzyme preparations. Enzymatic activity without inhibitor was used as positive control. Three negative controls, enzyme with no substrate, substrate without enzyme, and buffer only, were included to monitor background readings.

Inhibition of general proteinase activity was measured using azocasein as substrate. Universal buffer pH 8.5 was used in this experiment. Five microliters of the enzyme extract was preincubated with 1 μ l inhibitor for 15 min at 30 °C before azocasein solution and buffer were added to the enzyme/inhibitor solution. Other procedures were the same as described above.

2.5. Zymogram analysis

Five microliters of salivary gland or gut enzyme solution was analyzed on a 10% Tris–glycine gel with 0.1% gelatin incorporated as substrate (Invitrogen, Carlsbad, CA). Five microliters of salivary gland enzyme extract, preincubated with aprotinin was also included in this test. In another experiment, a 12% Tris–glycine gel (Invitrogen) with casein incorporated was used to compare enzymatic activities of salivary glands and gut. Each sample was mixed with an equal volume of 2 \times sample buffer (0.125 mM Tris–hydrochloride pH 6.8, 20% glycerol, 4% SDS, 0.005% bromophenol blue) and incubated for 10 min at room temperature (~21 °C). Following electrophoresis, enzymes were renatured in 2.5% Triton X-100, and the gel was developed for 4 h at 37 °C. The developed gel was then stained and destained following manufacturer's instructions.

2.6. Cloning trypsin-like protein cDNA

Approximately 50 salivary glands and guts were dissected from adult *L. lineolaris*. Total RNA was isolated separately from these tissues using TRIzol reagent (Invitrogen) following manufacturer's instructions. First-Strand cDNA was synthesized from total RNA with an oligo-dT primer using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). To obtain trypsin-like cDNA, PCR was conducted to amplify a cDNA

Table 1
Effect of selected proteinase inhibitors on the hydrolysis of azocasein or BApNA by *Lygus lineolaris* gut or salivary gland proteinases

Inhibitors	Concentration	Target enzyme	Percent inhibition ^a of azocaseinase activity		Percent inhibition ^a of BApNAse activity	
			Gut	Salivary glands	Gut	Salivary glands
Aprotinin	10 μ M	Serine protease	24 def	80 h	1 cd	81 ef
PMSF	5 mM	Serine protease	37 g	74 g	18 e	90 fg
STI	100 μ M	Chymo-/trypsin	28 ef	62 f	–10 a	40 d
Chymostatin	10 μ M	Chymotrypsin	30 fg	10 b	13 de	–15 a
TPCK	1 mM	Chymotrypsin	–5 a	24 c	4 cd	–9 ab
TLCK	1 mM	Trypsin	8 bc	29 d	59 f	48 d
Leupeptin	200 μ M	Serine/cysteine	15 cd	49 e	60 f	82 ef
E-64	100 μ M	Cysteine	6 b	30 d	12 de	16 c
Pepstatin	2 μ M	Aspartic protease	6 b	1 a	8 cd	2 b
Benzamidine	10 mM	Arg. bind pocket	46 h	80 h	19 e	79 ef
PCPI	5 μ M	Carboxypeptidase	21 de	65 f	–7 ab	76 e

^a Means followed by same letters within each column are not significantly different ($P \leq 0.05$).

fragment with a forward degenerate primer, 5'-CARMGNATHGTNGGNGG-3' and a reverse degenerate primer 5'-ARNGGNCCNCCNSWRGTGNCCYTGRCA-3', designed from two highly conserved regions of serine proteinases (QRIVGG and CQGDSGGPL, respectively) in both *Manduca sexta* trypsin and chymotrypsin cDNAs (Peterson et al., 1994, 1995). PCR-amplified DNA fragments (~600 bp) were cloned into a pGEM-T vector (Promega, Madison, WI). Sequence of the clone was determined by using an automated sequencer (Applied Biosystem Model, 3700). cDNA sequence of the trypsin-like protein was confirmed by homology search of GenBank provided by the National Center for Biotechnology Information using the Blastx protocol (Altschul et al., 1997).

5'-RACE (rapid amplification of cDNA end, Gibco BRL Life-Technologies) was performed to obtain the 5'-end of the trypsin-like precursor cDNA. Three reverse primers were designed from the cloned fragment. One reverse primer was used for reverse transcription, and the other two reverse primers were used with a forward abridged anchor primer for PCR and subsequent semi-nested PCR amplifications. Resulting cDNA fragments were purified and cloned into a pGEM-T vector. To obtain full-length trypsin cDNA, one specific forward primer was synthesized for flanking to a 5'-untranslated region (UTR) and was used in PCR amplification with oligo-dT primer. PCR amplification with Pfu DNA polymerase (Promega) was conducted to obtain full trypsin-like precursor cDNA from RT cDNA template. The resolved clone was sequenced from both directions.

2.7. Data processing and sequence analysis

Statistics Analysis System (SAS version 8, SAS Institute, Cary, NC) was used to analyze experimental data. The BLASTX program was used to search the sequence database of the National Center for Biotechnology Information Internet server for proteins with amino acid sequence similarity to the proteinases (Altschul et al., 1997). The ExpASY Proteomics tools of the ExpASY Molecular Biology Server of Swiss Institute of Bioinformatics (<http://www.expasy.ch/tools/>) were used to process data of deduced protein sequences. ClustalW (gap weight = 5, gap length weight = 1) was used for pairwise sequence comparison. BioEdit (Ver. 5.09, Hall, 1999) program was used to conduct multiple sequence alignment and phylogenetic analysis.

3. Results

3.1. Influence of pH on proteinase activity

The effect of pH on *L. lineolaris* gut proteinase activity was measured using azocasein and BApNA as

substrates (Fig. 1A). General proteinase activity, as measured by casein hydrolysis, was low at pH 2 but increased sharply to a maximum at pH 4.25. Azocaseinase activity decreased as the pH increased to 8.5 and was low at values above 8.5. Gut BApNAse activity was low below pH 5.3 and reached a maximum at pH 8.5. At pH values greater than 8.5, the activity gradually decreased.

General proteinase activity from *L. lineolaris* salivary glands was low below pH 4.25 (Fig. 1B). A sharp increase of the proteinase activity was observed over a pH range from 5.3 to 6.2. Azocaseinase activity reached and maintained a broad maximal level between pH 6.2 and 10.6. A sharp decline of the activity began as the pH increased to 11.1 and dropped to a very low level at pH 11.7.

3.2. Sensitivity of proteinases to inhibitors

The responses of *L. lineolaris* salivary gland and gut proteinases to 11 inhibitors are summarized in Table 1. The general proteinase activity in the salivary glands was effectively suppressed by several inhibitors. Among the inhibitors tested, aprotinin and benzamidine exhibited

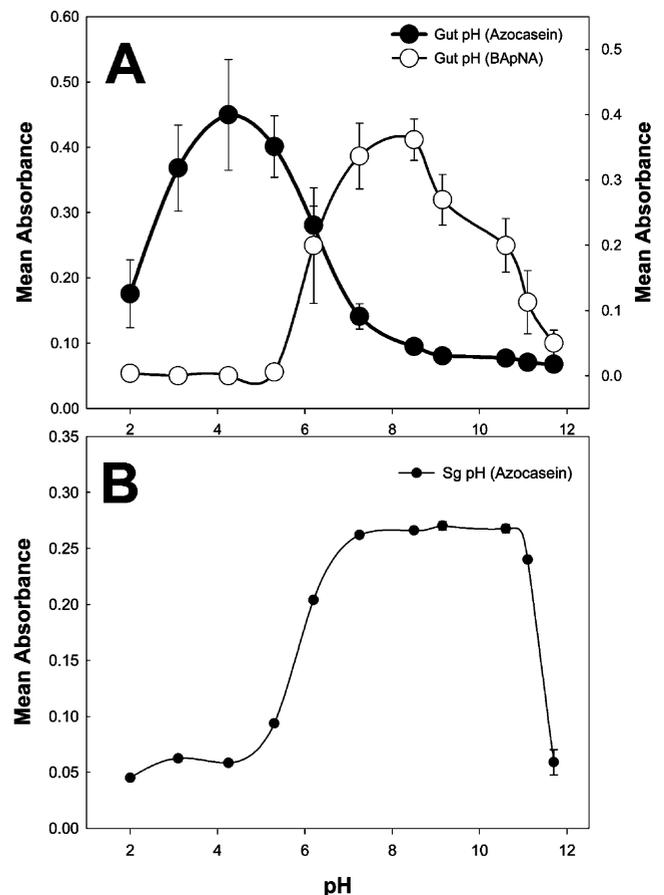


Fig. 1. (A) Effect of pH on azocaseinase (left axis) and BApNAse (right axis) activities of *L. lineolaris* gut proteinases. (B) Effect of pH on azocaseinase activity of *L. lineolaris* salivary gland proteinases.

the highest inhibition (80%) against azocaseinase activity in the salivary glands, followed by PMSF (74%), PCPI (65%), and STI (62%). Leupeptin showed moderate inhibition (49%), followed by E-64 and TLCK (~30%). The other inhibitors decreased general proteinase activity in the salivary glands by less than 25%. Inhibition of general proteinase activity in the gut was relatively lower than in the salivary glands. Benzamidine suppressed azocaseinase activity by 46%, and it was the most effective inhibitor of gut proteinases. However, it was only one-half as effective with gut proteinases as the salivary gland proteinases. PMSF, chymostatin, and STI had some effect (~30% inhibition) on gut proteinase activity. All other inhibitors showed less than 25% effectiveness against general proteinase activity in the gut.

The BApNase activity in the salivary gland was more susceptible to many inhibitors than azocaseinase activity (Table 1). PMSF was the strongest inhibitor (90% inhibition), followed by leupeptin, aprotinin, benzamidine, and PCPI with approximately 80% inhibition. TLCK and STI exhibited moderate inhibition, and the other inhibitors showed relatively low effectiveness (less than 20%). Inhibition of BApNase activity in the gut was less than that in salivary glands. The most effective inhibitors of BApNase activity were leupeptin and TLCK, and less than 20% of the activity was affected by the other inhibitors.

3.3. Proteinase zymograms

On a gelatin-containing zymogram, a clear area was formed when the gelatin was digested by proteinases, where the undigested gelatin stained blue. Proteinases from salivary glands digested gelatin, resulting in a clear band of enzyme activity with a molecular mass of approximately 26 kDa (Fig. 2A, Sg). Proteinases from

the gut did not digest gelatin (Fig. 2A, Gut). Salivary glands proteinase activity was inhibited by aprotinin (Fig. 2A, Sg+A).

On a casein-containing zymogram, proteinases from gut tissues were not active (Fig. 2B, Gut). However, two caseinolytic proteinases from salivary glands, approximately ~30 and 26 kDa, were detected (Fig. 2B, Sg).

3.4. Trypsin-like protein cDNA

By using RT-PCR and 5'-RACE, more than 20 clones were successfully sequenced from the salivary glands and gut of *L. lineolaris*. Several of these clones were identical. In addition to a trypsin-like cDNA previously reported (Zeng et al., 2002a; designated as LISgP1 here), three additional trypsin-like cDNAs, LISgP2, LISgP3, and LISgP4, were cloned from salivary glands and one trypsin-like cDNA, LIGtP1 was cloned from gut (Figs. 3 and 4).

LISgP2 cDNA consisted of 956 nucleotides with a 873 bp open reading frame (ORF) (Fig. 3). A total of 9 nucleotide differences were found in the ORF, four of which resulted in three amino acid changes between LISgP1 and LISgP2. LISgP3 cDNA had 954 nucleotides. A 873 bp ORF had the same length as LISgP1 and LISgP2. There were eight nucleotide differences in the ORF between LISgP1 and LISgP3 and only one nucleotide resulted in a translated amino acid difference. Fifteen nucleotides in LISgP3 differed from LISgP2 and resulted in four amino acid differences.

LIGtP1 was amplified from gut RT-cDNA with two primers flanking to each end (Fig. 3). The total 905 bp gut cDNA contained the same length ORF (873 bp) as the other three cDNAs (LISgP1–3). Eleven nucleotide differences caused three amino acid variations between LIGtP1 and LISgP1. Nineteen nucleotide differences resulted in six amino acid changes between LIGtP1 and LISgP2. Fourteen nucleotides were different between LIGtP1 ORF and LISgP3 ORF, and four of them coded four different amino acids.

LISgP4 cDNA contained a total of 1218 nucleotides (Fig. 4) with a 897 bp ORF. LISgP4 had a 298 bp 3'-UTR, more than 200 bp longer than other cDNAs cloned from salivary glands. It had a poly-A tail but lacked a conserved AATAAA polyadenylation signal. Pairwise alignment (GapOpen = 5, GapExt = 1) sequence comparison revealed only 54.42% sequence identity between ORFs of LISgP4 and either of the other three (LISgP1, LISgP2, LISgP3).

3.5. Deduced protein sequences

GenBank homology search indicated that the deduced amino acid sequences from the three new clones from salivary glands and one clone from the gut of *L. lineolaris* were similar to trypsin-like proteins. Those highly

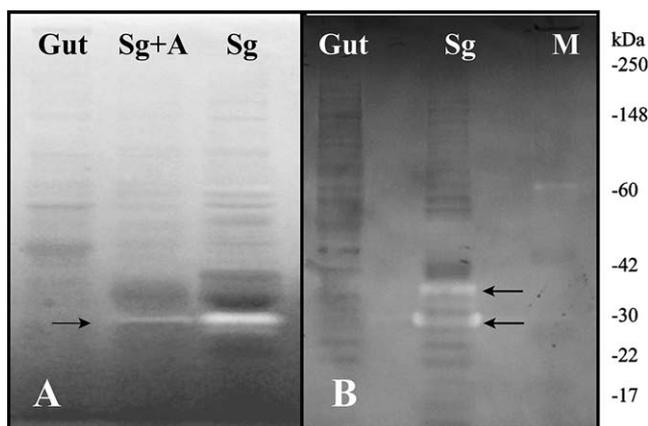


Fig. 2. (A) Comparison of gut (Gut) and salivary gland (Sg) proteinase activity on 10% Tris–glycine gel with 0.1% gelatin incorporated as a substrate. Sg + A=proteinases from salivary glands preincubated with 1 μ l aprotinin (1 mM). (B) Comparison of gut and salivary gland proteinase activity on 12% Tris–glycine gel with beta-casein incorporated as a substrate. M = MultiMark (Invitrogen). Arrows indicate clear bands where the substrates were digested by proteinases.

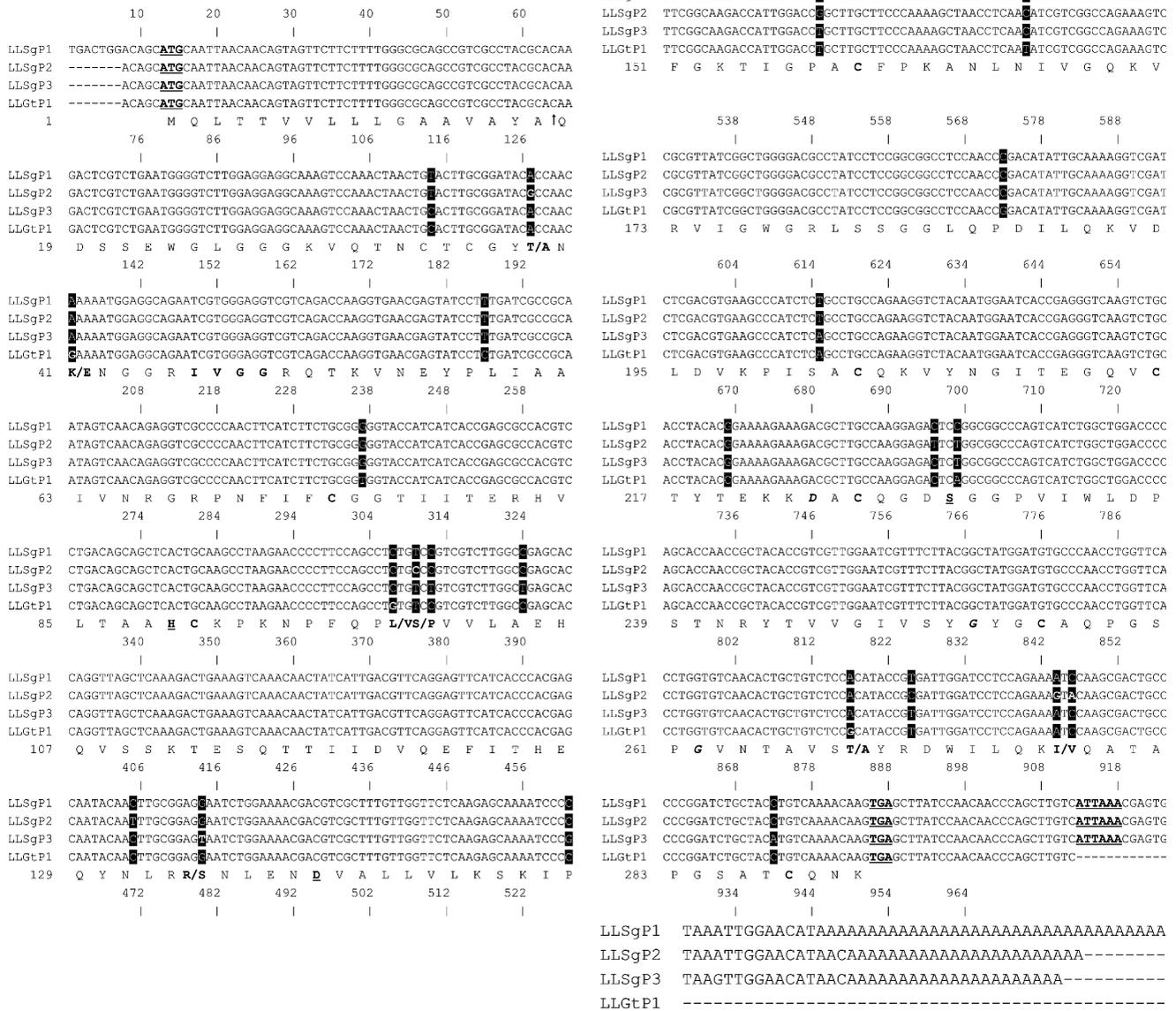


Fig. 3. Nucleotide and deduced amino acid sequences of trypsin-like cDNAs aligned with LLSgP1 cloned previously from salivary glands of *L. lineolaris* (GenBank: AF414430; Zeng et al., 2002a). LLSgP2 and LLSgP3: isolated from salivary glands of *L. lineolaris*; LLGtP1: isolated from gut of *L. lineolaris*. ATG = start codon; TGA = termination codon; ATTA = potential polyadenylation signal; | = predicted signal peptide cleavage site between residue 17 and 18; IVGG = conserved N-terminal residues. Catalytic triad active site residues are indicated as H, D, and S. Binding pocket residues are indicated as D, G, and G. Eight cystine residues are in bold. Codons and nucleotide differences among four trypsin-like cDNAs are highlighted with black background, and corresponding amino acid changes are indicated with substitute residues following slash (/). The GenBank accession number: AY190925 for LLSgP2; AY190926 for LLSgP3; AY190928 for LLGtP1.

matched proteins included a trypsin precursor from *L. lineolaris* (designated as LLSgP1 here; Zeng et al., 2002a), a trypsin precursor from the western tarnished plant bug, *L. hesperus* (designated as LhSgP1 here; Zeng et al., 2002b), and a serine proteinase CdSP1 from the green mirid, *Creontiades dilutus* (Colebatch et al., 2002).

LLSgP2, LLSgP3, and LLGtP1 cDNA coded a 291 amino acid trypsin-like precursor with molecular mass of approximately 31 kDa and pI 9.04. All three deduced trypsin-like precursors contained a 17-residue signal peptide predicted using Signal 1P software (Nielsen et al., 1997), and a 28-residue activation peptide. Mature

trypsins had a conserved IVGG N-terminus, 25–26 positively charged residues, 17–18 negatively charged residues, and molecular mass of approximately 26.7 kDa and pI 9. Four, five, and three S/T-xx-D/E sequence motifs for casein kinase II phosphorylation sites were predicted from mature trypsins deduced from LLSgP2, LLSgP3, and LLGtP1, respectively.

LLSgP4 cDNA from salivary glands had a longer ORF, and encoded a 299 amino acid trypsin-like precursor with a molecular mass of 33.29 kDa and pI 7.57. The precursor was predicted to consist of a 16-residue signal peptide and 33-residue activation peptide (Fig. 4).

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AGTCATTTTCGGTTGAACAAGAAGA 26
ATGAGAGTTTGGATTCCCGCTATTTTGTATTTCCTCGTCGGCGCTTTTCTGAAGACGATGTGAGTGAA 92
M R V W I P L F C I L V A A F A E D D V S E 22
GACTCCTCGGAGCATGGAGTCGTCAAGGGGGGAAAGAACCAACTGCCGATCGCGCTGGCCAAAT 158
D S S E H G V V K G A K G T N C R C G W A N 44
AAAGACAGTCAAAGGATTGTGGAGGCAAGAAACAAAAGTGAACGAGTATCCTATGATGCTGGGA 224
K D S Q R I V G G K E T K V N E Y P M M A G 66
TTATTTTACACTCCGAGGAATGTTTGTGTTTTCGGAGGTACCGTGATACAGGTGGCACGTTGTA 290
L F Y T P R N V L F C G G T V I T R W H V 88
ACTCAGCACACTGTGTTGAACCTGTTCCATGTGCCAAGACGTTCAAATGTCTTAGGAGAA 356
T A A H C V E P V L H V P E D V Q I V L G G E 110
CACGACCAAGCAAGTGTGATGAATCGCCGACACCAAGGTTTACAGAGTAAAGAAATGGTAAT 422
H D Q S K V D E S P Y T K V Y R V K E M V N 132
CATCCAGATTTTCTCGGTTGGACACAGAAATGATATTGCGTTCATCCTGTCGAGACGAGATTT 488
H P D Y F L V G H R N D I A V I L S E T R F 154
GAATTCACAGTATGTGGTCCCTGCTGCATGCTACTGGAGCTGAAGTCATAGTTGGTTCCAAA 554
E F N D Y V G P A C A K V Y D R V N T T E A 176
CTGAAGTTTATAGTTGGGAAACTCTCTGTAGATGGACAGCTTCCAAAGTACTGATGAAAGTA 620
L K V L G W G K L S V D G P A S K V L M K V 198
AATCTGAATGATGTTCCAATGAAACATGCCAAAGTATACGACAGGTTAAACACGACTGAGGCG 686
N L N V V P I E Q C A K V Y D R V N T T E A 220
AAACAAGTGGCACTCATCATCAAGAAAGATGCTGTGTCAGGAGATTCGCGGTCCTCAGCTGC 752
K Q V C T H H P R K D A C Q G D S G G P L L 242
TGGCTTGACAGAGAACAATAGATACACATTTGGTAGTCTGCTTTCTCATGTTAGAGGCTGTGCC 818
W L D R E T N R Y T L V A A V S H G R G C A 264
GAAGGTCATCCAGCTGTCACACCAATATTTACTACAGGGACTGGATCAAAAATATTACAGA 884
E G H P A V N T N I I Y R D W I Q K I R 286
GAAACGAAGCCAGAAGGATTGCAAGAAACAGGGTTAATCATCAGTCTTTTCATGTGGTGAC 950
E T K P E G R I C K K Q G 299
CGCTAGGAGCGCAGCAGGGGCTCGAGACTCTAGAAGTGTCTCCCTCCACTCATAGTGAACAGAAA 1016
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TACGAATGCTGATAGAAAAATGATTACATACCTGAAGATTTTACCTATAAGAAATCAATGAGTTGG 1148
AAAAATATACCTTTGTTTTCGAGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAAAGAAAAA 1214
AAAA 1218
    
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Fig. 4. Nucleotide and deduced amino acid sequences of trypsin-like cDNA LISgP4 cloned from salivary glands of *L. lineolaris*. Nucleotide and amino acid denotations refer to Fig. 3 legend. The GenBank accession number: AY190927 for LISgP4.

Mature trypsin-like protein also had a conserved IVGG N-terminus, 30 positively charged residues, and 28 negatively charged residues. The predicted molecular mass for mature protein was 27.90 kDa, and pI was 8.12. Four S/T-xx-D/E sequence motifs for casein kinase II phosphorylation sites were predicted.

3.6. Sequence comparison and analysis

A total of seven proteinase sequences were aligned (Fig. 5). These trypsin and chymotrypsin-like precursors were cloned from three mirid species, including one previously reported trypsin precursor LISgP1 from SGC of *L. lineolaris* (Zeng et al., 2002a), one trypsin precursor LhSgPI from SGC of *L. hesperus* (Zeng et al., 2002b), one chymotrypsin precursor CdSP1 from *C. dilutus* (Colebatch et al., 2002), three trypsin-like precursors, LISg2–4, from SGC and one trypsin-like precursor from gut of *L. lineolaris* in this study. These trypsin or chymotrypsin-like precursors contained the conserved IVGG sequence that is preceded by an arginine or lysine for most serine proteinases. In this alignment, His⁹⁵, Asp¹⁴⁸, and Ser²⁴², the three putative active site residues, were conserved in all seven mirid serine proteinases (numbers

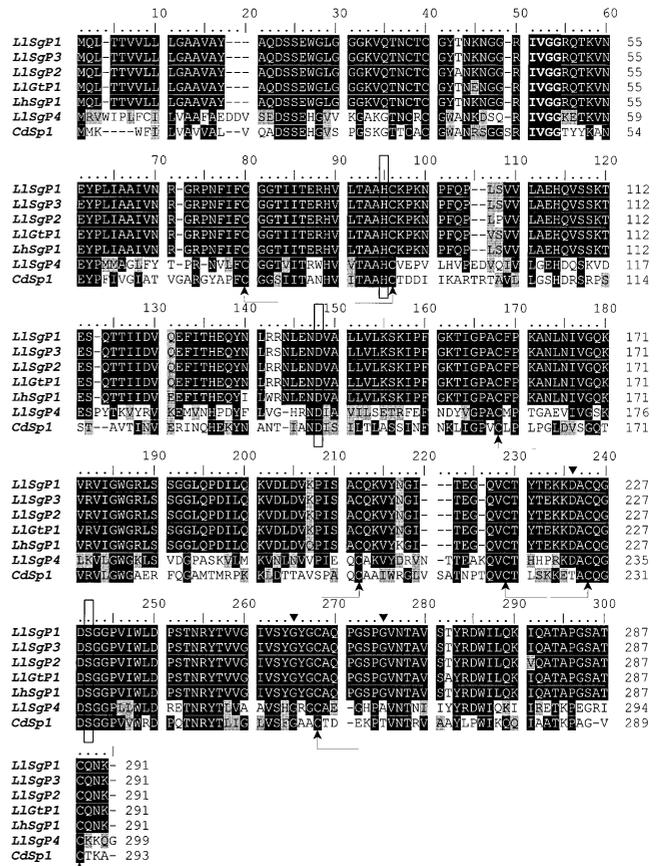


Fig. 5. Multiple alignment of predicted amino acid sequences of five trypsin-like precursors from *L. lineolaris*, one trypsin-like precursor from *L. hesperus* (GenBank: AF356841, Zeng et al., 2002b), and a chymotrypsin precursor from *C. dilutus* (GenBank AAL15154, Colebatch et al., 2002). N-terminal residues IVGG are in bold. Functionally important residues H, D, and S are boxed. Cysteines corresponding to the sites of the predicted disulfide bridges are marked with arrow lines at the bottom. Trypsin specificity determinant residues are indicated with (▼) on the top of sequences. Identical or highly similar residues among all seven sequences are highlighted with black or gray background. Hyphens represent sequence alignment gaps.

are assigned based on total number of 305 residues of multiple alignment of seven insect proteinases). Eight cysteine residues that were conserved in all of these mirid proteinases were located at positions 80, 96, 168, 212, 229, 238, 268, and 301. These cysteine residues are predicted to occur in disulfide bridge configurations (Wang et al., 1993).

Results from ClustalW phylogenetic analysis (Table 2) indicated that these mirid proteinases formed three groups. LISgP1, LISgP2, LISgP3, LIgT1, and LhSgP1 were grouped together and located on one branch. CdSp1 had greatest distance from other trypsin-like proteinases of *Lygus* species, and was located on an independent branch. LISgP4 was located on another independent branch, which was distant from other trypsin-like proteinases cloned from the same species, *L. lineolaris*.

Table 2
Phylogenetic distance among serine proteinases cloned from Miridae

Sequence name	Insect species	GenBank accession	Phylogenetic distance					
			LiSgP3	LiSgP2	LiGtP1	LhSgP1	LiSgP4	CdSp1
LiSgP1	<i>L. lineolaris</i>	AF414430	0.00338	0.00880	0.01018	0.01707	0.94673	1.06168
LiSgP3	<i>L. lineolaris</i>	AY190926		0.01360	0.01358	0.02049	0.93946	1.05363
LiSgP2	<i>L. lineolaris</i>	AY190925			0.02049	0.02748	0.94388	1.06321
LiGtP1	<i>L. lineolaris</i>	AY190928				0.02744	0.94682	1.05769
LhSgP1	<i>L. hesperus</i>	AF356841					0.95636	1.06663
LiSgP4	<i>L. lineolaris</i>	AY190927						1.17457
CdSp1	<i>Creontiades dilutus</i>	AAL15154						0.00000

4. Discussion

4.1. Biochemical aspects of proteinases in *L. lineolaris*

In this study, we observed differences in the relative activities of proteinases from the salivary glands and those from the gut of *L. lineolaris*. Caseinolytic proteinases from the salivary glands had neutral to alkaline pH optima in a broad range, whereas gut proteinases displayed maximal activity at a very low pH levels with a narrow range. Optimal pH for the hydrolysis of BApNA by gut proteinases differed from the hydrolysis of azocasein. BApNAse activity was maximum at pH 8.5, significantly different from the pH 10 optima observed in the hydrolysis of BApNA salivary gland proteinases (Zeng et al., 2002a). Different pH optima suggest different proteinases in the gut and salivary gland. Alkaline proteinases may predominate in the salivary glands for protein digestion, similar to what has been reported for many Lepidoptera insects (Ortego et al., 1996; Novillo et al., 1999; Peterson et al., 1994). Gut proteinases may include not only alkaline proteinases, but other proteinases that have optimal activity in neutral or acidic conditions, such as cysteine proteinases that have been observed in some coleopteran insects (Girard et al., 1998; Michaud et al., 1995; Terra and Cristofolletti, 1996).

We demonstrated that the general proteinase activities in the salivary glands and gut of *L. lineolaris* were reduced by many different inhibitors, especially those targeting serine proteinases. More than 70% of the general proteinase activity and 90% of BApNAse activity in salivary glands were inhibited by PMSF, and approximately 80% of both azocaseinase and BApNAse activities were suppressed by aprotinin. These results indicated that up to 80% of proteinase activities in the salivary glands resulted from serine proteinases. STI inhibits chymotrypsin and trypsin, and it effectively reduced the general proteinase activity of the salivary glands by more than 62%. TPCK and chymostatin

specifically target chymotrypsin, but neither inhibitors were effective against salivary gland proteinase activity. These results suggest that trypsin-like proteinases are responsible for protein digestion in the salivary glands. However, the trypsin inhibitor, TLCK, suppressed general proteinase activity by only 30% and BApNAse activity by ~48%. It is possible that the concentration of TLCK (1 mM) was not high enough to suppress the BApNAse activity in the salivary glands of *L. lineolaris*, although this same level inhibited midgut BApNAse activity of *Rhyzopertha dominica* (F.) by 94% (Zhu and Baker, 1999).

Analyses with leupeptin, which targets both serine and cysteine proteinases, indicated that cysteine proteinases contributed less than 9% to general proteinase activity (15% total inhibition) because ~40% of general proteinase activity came from serine proteinases (37% inhibition by PMSF). Another cysteine proteinase inhibitor, E-64, suppressed approximately 30% of the general proteinase activity and 16% tryptic activity of salivary gland proteinases. Non-specific inhibition of serine proteinases by E-64, observed by Sreedharan et al. (1996), also indicated that cysteine proteinase activity was less than serine proteinase activity in the salivary glands. The lack of inhibition of azocaseinase activity by pepstatin suggested that aspartic proteinases were absent in the salivary glands. Benzamidin, which inhibits proteinases with an arginine binding pocket, had approximately the same effect on general and tryptic activities of the salivary glands. PCPI, a carboxypeptidase inhibitor, showed relatively high inhibition of both general and tryptic activities (65%, 76%), suggesting that carboxypeptidase and trypsin activities are linked.

Proteinases from the gut were less sensitive to inhibitors than those from the salivary glands. Benzamidin was the most effective gut proteinase azocaseinase inhibitor. Although other serine proteinase inhibitors were less effective against gut proteinases, inhibition data still indicated that serine proteinases are predominant in the gut. Chymotrypsin may play a minor role in gut digestion, as 13% of the gut BApNAse activity was

inhibited by chymostatin. However, another chymotrypsin inhibitor, TPCK, did not inhibit gut proteinases. Trypsins appear to be the most abundant proteinases in the gut of *L. lineolaris*, due to the inhibition by TLCK and leupeptin. In addition to serine proteinases, the gut also may contain other proteases, such as cysteine proteinases and carboxypeptidases, which under normal dietary conditions are present at relatively low levels in gut. The composition of gut proteinases may be altered by the transfer of salivary gland enzymes into the gut with the food slurry (Cohen (1993, 1998).

Previously, we found that the serine protease inhibitors, PMSF and lima bean trypsin inhibitor, completely inhibited protease activity when analyzed on casein zymograms (Zeng et al., 2002a). In this study, we demonstrated that gelatin and casein were readily digested by proteinases from salivary glands. The molecular mass of the proteinases, as determined by zymogram gels, were similar to trypsin, and their activity was reduced by aprotinin. Gut proteinases were unable to digest gelatin and casein to produce distinct bands on zymogram gels, although they occasionally generated bands around 100 kDa size and were easily masked by background during staining and destaining processes (data not shown).

4.2. Molecular aspects of digestive proteinases

Previously, we cloned a trypsin-like cDNA from salivary glands of *L. lineolaris* (Zeng et al., 2002a). In this study, we cloned three additional trypsin-like cDNAs from the salivary glands and one trypsin-like cDNA from guts. Predicted protein sequences from all of these cDNAs shared common features to trypsin-like proteins. These included a signal peptide, an activation peptide, and a highly conserved N-terminus (IVGG) found in many trypsin- and chymotrypsin-like proteinases, which marks the N-termini of the active enzymes (Jany and Haug, 1983; Wang et al., 1993). Three important residues His⁹⁵, Asp¹⁴⁸, and Ser²⁴² are conserved to form the catalytic triad in serine proteases (Kraut, 1977; Wang et al., 1993; Peterson et al., 1994). Other important residues included D²³⁶, G²⁶⁵, and G²⁷⁵, which define the substrate binding pocket also were highly conserved in all of these trypsin-like enzymes except A²⁷⁵ in LISgP4. The D²⁶³ determines specificity in both invertebrate and vertebrate trypsin by stabilizing the Lys or Arg residue at the substrate cleavage site through ionic interactions (Hedstrom et al., 1992; Wang et al., 1993).

Three pairs of cysteine residues that are configured as disulfide bridges are commonly present in trypsin and chymotrypsins. An extra cysteine may occur in some insects (Jongsma et al., 1996; Zhu and Baker, 1999). In *L. lineolaris*, eight cysteine residues are present in all the trypsin-like proteins. This extra pair of cysteines was also found in a trypsin-like protein of the closely related species, *L. hesperus* (Zeng et al., 2002b) and in a chymo-

trypsin-like protein of *C. dilutus* (Colebatch et al., 2002). Trypsins with four cysteine pairs instead of three might be a characteristic of the Hemiptera insects, but the pairing mode of cysteine residues for disulfide bridge configurations in this insect has yet to be established. It is very likely that trypsin in these hemipteran species have free cysteine residues, and these free cysteines might be responsible for the vulnerability of the enzymes to some typical thiol-reacting inhibitors (Jongsma et al., 1996; Gatehouse et al., 1997). This is one explanation for the inhibition of salivary gland BApNAse activity by E-64.

L. lineolaris has more than 300 host plant species (Young, 1986). Adaptation to a wide range of food sources may be facilitated by evolving a variety of proteinases to digest different proteins. Multigene trypsin families may have evolved to provide a more efficient mechanism for protein digestion as well as to provide an adaptive advantage for phytophagous species feeding on plants that contain proteinase inhibitors (Bown et al., 1997; Reeck et al., 1999). Like *Manduca sexta* (Peterson et al., 1994) and *Anopheles gambiae* (Müller et al., 1993), *L. lineolaris* also has evolved with a variety of trypsin.

In this study, we cloned four additional trypsin-like cDNAs from salivary glands and guts. Based on phylogenetic analysis, deduced trypsin shared 98–99% sequence identity among LISgP1–3, LIGtP1, and LhSgP1. LISgP1–3 and LIGtP1 may have evolved from common ancestors and subsequently diverged through gene duplications as observed in dipterans and lepidopterans (Wang et al., 1995). Surprisingly, LISgP4, from salivary glands, shared only 44% sequence identity with the other trypsin-like proteinases from the salivary glands and gut of *Lygus* species. Low sequence identity resulted in clear separation from the other trypsin-like proteinases, suggesting that LISgP4 may have evolved independently. This might also be an explanation for the difference in inhibitor specificity observed between salivary gland and gut proteinases.

Besides cDNAs encoding four trypsin-like proteinases, LISgP1–4, from salivary glands, we also obtained a partial proteinase-like cDNA from salivary glands (data not shown). The deduced protein sequence differed from any sequence reported in this paper but it was highly similar to LISgP1 or LhSgP1 with 36% partial sequence identity. An attempt to clone the full cDNA was not successful, possibly due to the lack of a poly-A tail or low mRNA expression levels in the salivary glands.

In conclusion, *L. lineolaris* has evolved sucking mouthparts to ingest plant juice. Extra-intestinal digestion is facilitated through injection of saliva containing variety of digestive enzymes into plant tissue. Proteins are critical nutrients for insect growth and development. This study was designed to understand and eventually

manipulate protein digestion in *L. lineolaris*. Proteinases, such as trypsin, play an essential function for protein digestion and absorption in *L. lineolaris*. The introduction of proteinase inhibitors, especially those that target trypsin, into host plants may substantially suppress protein digestion, and subsequently achieve insect control through nutrient deficiency. We demonstrated that protein digestion in *L. lineolaris* depended primarily on proteinases from salivary glands, followed by gut proteinases for further digestion. Serine proteinases, especially trypsin, are the major proteinases in both salivary glands and guts. Protein digestion was affected by pH and was suppressible by many serine proteinase inhibitors.

5. Sequence registration

The sequences have been deposited in the GenBank with accession number AY190925 for LISgP2; AY190926 for LISgP3; AY190927 for LISgP4; AY190928 for LIgtP1.

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