

Association of midgut defensin with a novel serine protease in the blood-sucking fly *Stomoxys calcitrans*

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Abstract

Using ELISA we provide direct evidence that the midgut defensins of the blood-sucking fly *Stomoxys calcitrans* are secreted into the gut lumen. We show that midgut defensin peptide levels increase up to fortyfold in response to a blood meal but not to a sugar meal. The data suggests the midgut defensin genes are post-transcriptionally regulated and that their function is protection of the stored blood meal from bacterial attack while it awaits digestion. Using recombinant defensins produced in *Pichia pastoris* we demonstrate that while in the gut cells the midgut defensins are bound in an SDS-stable complex to proteins with an apparent molecular weight of > 26 kDa from which they are released when secreted into the gut lumen. This > 26 kDa protein (Ssp3) has been cloned and sequenced and is a member of the serine protease S1 family with homologies to multiple insect proteases and to vertebrate trypsins and elastases.

Keywords: defensin, serine protease, immunity, blood-sucking, midgut.

Introduction

The major immune responses of insects, or at least the most studied, are mounted from the insect fat body. Challenge to the insect leads to transcriptional up-regulation of a series of genes and the *de novo* synthesis and secretion of a battery of immune peptides from the fat body (Hoffmann & Reichhart, 1997). These insect immune responses are non-clonal and have many similarities to the

innate immune system of vertebrates (Medzhitov & Janeway, 1997). Insects also have well developed epithelial immune responses (Brey *et al.*, 1993; Lehane *et al.*, 1997; Tzou *et al.*, 2000). In *Drosophila* all of the epithelial surfaces of the insect body produce antimicrobial peptides usually as a subset of the total number of antimicrobial peptides in the insect; the subset is usually complementary containing both anti-Gram positive and anti-Gram negative activities (Tzou *et al.*, 2000). Among these epithelia only *Drosophila* midgut expresses all of the antimicrobial peptides (Tzou *et al.*, 2000) and this reflects the relative vulnerability of the gut to infection, which is common to all metazoa. Given that the midgut is the primary site of entry for the most important insect borne parasites including malaria, trypanosomes and arbovirus for example, it is surprising how little we know about insect midgut immunity, particularly in blood-sucking insects. We do know that agglutinins play an antiparasitic role in the midgut of some insects (Maudlin, 1991), that peritrophic matrix has a defensive function (Lehane, 1997), that the anti-Gram positive enzyme lysozyme may be present and that *Plasmodium* ookinetes may, on occasion, be lysed by undefined mechanisms in the midgut (Vernick *et al.*, 1995).

In blood-sucking insects secretion of antimicrobial peptides in the midgut epithelium has been demonstrated in *Stomoxys calcitrans* (Lehane *et al.*, 1997), *Anopheles gambiae* (Dimopoulos *et al.*, 1997) and *Glossina morsitans morsitans* (Hao *et al.*, 2001). The two midgut specific defensins reported from the anterior midgut (reservoir) of the stable fly *S. calcitrans* were particularly unusual in being specific to that tissue and being constitutively produced (Lehane *et al.*, 1997; Munks *et al.*, 2001). Those studies were performed exclusively on mRNA from these genes. Consequently there is no direct proof that those defensin peptides are indeed used in the midgut lumen. Also there is mounting evidence that many genes in the midgut of blood-sucking insects are post-transcriptionally controlled so that meaningful data on gene product levels can only come from studies of the peptides themselves (Muller *et al.*, 1995; Lehane *et al.*, 1998; Noriega & Wells, 1999). In this study we set out to use protein-based techniques to obtain a clearer view of the biology of the *S. calcitrans* midgut defensins.

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Results

Recombinant proteins

The *Pichia* system produced low levels of recombinant protein; range 0.1–16.7 µg/l for Smd1 and 0.6–6.2 µg/l for Smd2. Mass spectrophotometric analysis (data not shown) suggests both recombinant defensins were modified compared to the native forms. Recombinant Smd1 was truncated losing its first four amino acids. Recombinant Smd2 was glycosylated (internal N-linked site at amino acid positions 12–14 in the mature peptide) whereas the native form is not (Lehane *et al.*, 1997). Changing expression medium from minimal methanol to a medium enriched with 1% casamino acids made no difference to either modification. Despite these structural changes from the native defensins these recombinant proteins provided useful positive controls in the antisera work as described below.

Specificity and cross-reactivity of Smd1 and Smd2 rabbit antisera

The specificity of the antisera was determined by Western blotting (Fig. 1) and ELISA (Fig. 2), the cross-reactivity of the two sera with each other was also determined in ELISA (Fig. 2). Western blotting with the sera against recombinant Smd1 and Smd2 demonstrated that each serum recognized a single band, at approximately 4 kDa, which corresponded with the predicted molecular weights of 4736 Da for Smd1 (Fig. 1A) and 4237 Da for Smd2 (Fig. 1B) (Lehane *et al.*, 1997). These protein bands were not recognized by either the control serum or the pre-immune sera (Fig. 1C, lanes 1 & 2 and 3 & 4, respectively).

In ELISA, checkerboard titration of antisera and antigen revealed that the optimum serum dilution was 1 : 5000 when an antigen coating concentration of 5 µg/ml was

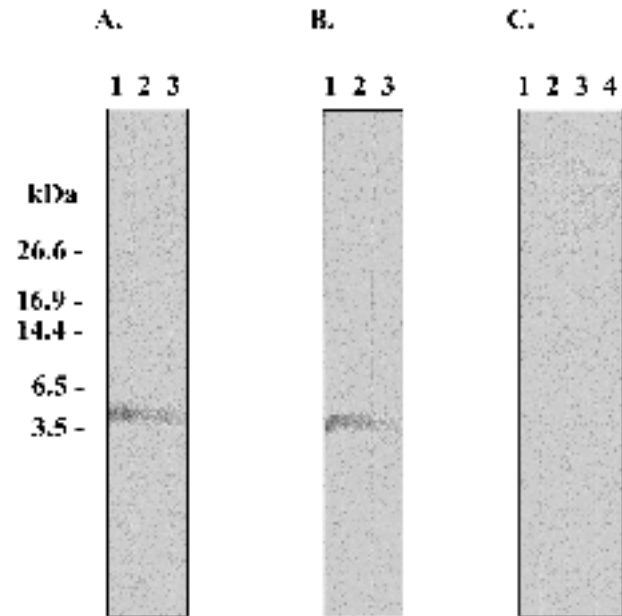


Figure 1. Specificity of Smd1 and Smd2 antisera. Western blot of 0.87 µg/lane of recombinant Smd1 (Panels A & C) or Smd2 (Panel B) peptide separated on 16.5%T, 3%C Tris-Tricine SDS-PAGE probed with A, anti-Smd1 sera (MJL3), 1 : 50 dilution (lane 1); 1 : 100 dilution (lane 2); 1 : 200 dilution (lane 3). B, anti-Smd2 sera (MJL8), 1 : 50 dilution (lane 1); 1 : 100 dilution (lane 2); 1 : 200 dilution (lane 3). C, control sera (MJL10) 1 : 50 dilution (lane 1); 1 : 100 dilution (lane 2). Pooled pre-immune sera 1 : 50 dilution (lane 3) and 1 : 100 dilution (lane 4).

used (data not shown). ELISA demonstrated that Smd1 and Smd2 antiserum recognized both the synthetic peptide against which they were raised (data not shown) and the recombinant Smd1 and Smd2 peptides (Fig. 2). Control and pre-immune sera were used to establish baseline background activity. In summary, the two sera used in this study were sensitive, specific and did not cross-react with each other (Figs 1 and 2).

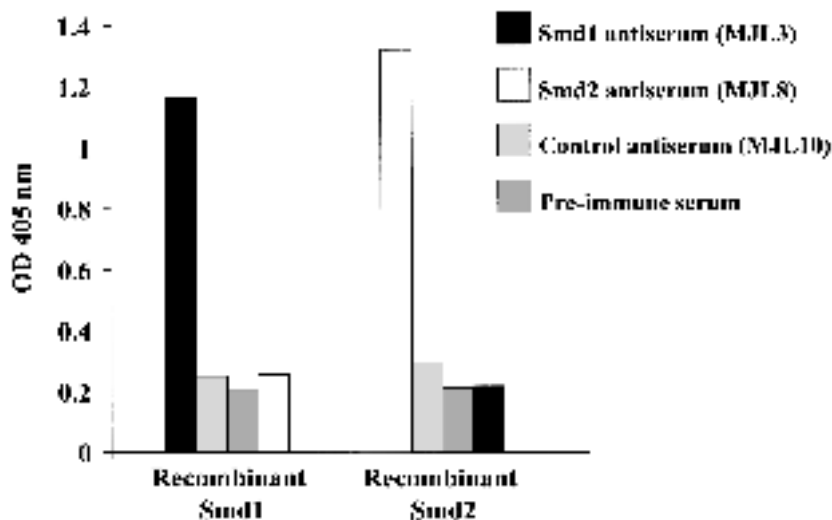
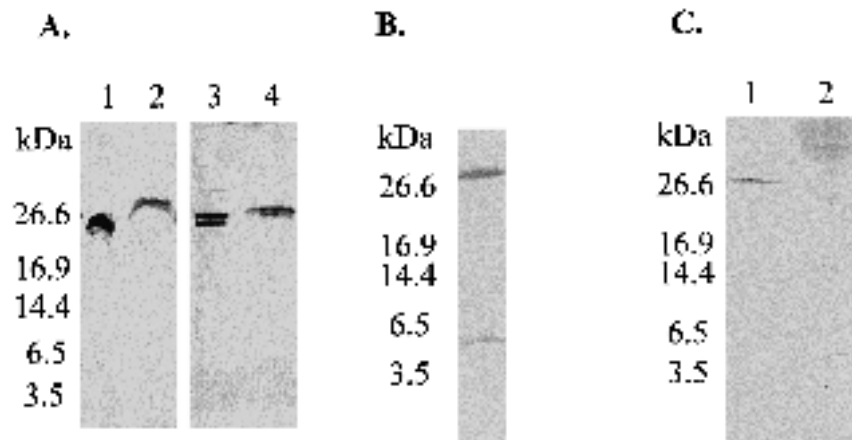


Figure 2. Sensitivity, specificity and cross-reactivity of Smd1 and Smd2 antisera in ELISA. Corrected ELISA optical densities, obtained as described in ELISA experimental procedures section. Graph 1: recombinant Smd1 peptide probed with Smd1 antiserum (MJL3, black bar), Smd2 antiserum (MJL8, white bar), control antiserum (MJL10, light grey bar) and pre-immune serum (dark grey bar). Graph 2: recombinant Smd2 peptide Smd2 antiserum (MJL8, white bar), Smd1 antiserum (MJL3, black bar), control antiserum (MJL10, light grey bar) and pre-immune serum (dark grey bar).

Figure 3. Colocalization of defensin and Ssp3 in the midgut of *Stomoxys calcitrans*. Western blots of midgut samples separated on 16.5%T, 3%C Tris-Tricine SDS-PAGE. A, effect of spiking whole reservoir zone homogenate with recombinant Smd1: 10 µg homogenate from unfed flies (lane 1); 10 µg homogenate from unfed flies spiked with 2.5 µg recombinant Smd1 peptide (lane 2); 10 µg homogenate from fed flies (24 h post blood meal) (lane 3); 10 µg homogenate from fed flies spiked with 2.5 µg recombinant Smd1 peptide (lane 4). B, presence of Smd1 defensin (= 4 kDa band) and Ssp3 (> 26 kDa band) in 50 µg reservoir zone homogenate from fed flies (24 h post blood meal). C, presence of Ssp3 (> 26 kDa band) in 10 µg reservoir zone tissue (lane 1) and Smd1 defensin (~4 kDa band) in 10 µg of reservoir zone contents (lane 2) from fed flies (24 h post blood meal).



SDS-stable complexes with Smd1 defensin

Western blotting of 10 µg per lane of reservoir zone homogenates revealed the presence of a > 26 kDa band(s) when probed with Smd1 antiserum (Fig. 3A, lane 1) and Smd2 antiserum (data not shown), but did not demonstrate the presence of the expected ≈ 4 kDa band (defensin). When these reservoir zone homogenates (10 µg per lane) were spiked with recombinant Smd1 peptide (2.5 µg per lane) and probed with Smd1 antiserum, the apparent molecular weights of these > 26 kDa bands increased (Fig. 3A, lanes 1 & 2). This was consistent for midgut homogenates from both unfed (Fig. 3A, lanes 1 & 2) and fed flies (Fig. 3A, lanes 3 & 4). In these 'spiking' experiments we did not observe the presence of the expected ≈ 4 kDa defensin band. When we increased the amount of midgut homogenate loaded on to the gels to 50 µg per lane and probed the blot with Smd1 antisera we detected the presence of both the > 26 kDa band and the ≈ 4 kDa defensin band (Fig. 3B).

After establishing the presence of Smd1 defensin in the midgut we repeated the experiment using flies dissected 24 h after a blood meal, but this time carefully separated the reservoir tissue from the reservoir contents. We now found that the > 26 kDa band was only present in the reservoir tissue (Fig. 3C, lane 1) whilst the 4 kDa band was only present in the gut contents (Fig. 3C, lane 2). These results coupled with the results from the 'spiking' experiment (Fig. 3A) led us to hypothesize that Smd1 peptide is able to associate in an SDS-stable complex with this higher molecular weight protein. Although no other specific defensin-protein SDS-stable complexes are described in the literature, other SDS-stable peptide-protein complexes are well known from vertebrate studies (Scott *et al.*, 1999).

The presence of a > 26 kDa doublet in some of the experiments may be due to the detection of a modified form of the > 26 kDa protein, for example glycosylation or other post-translational modifications, or may simply be due to

incomplete reduction of disulphide bonds during sample preparation. It is also possible that there may be some antibody cross-reactivity, either specific cross-reactivity of a similar or related protein or non-specific cross-reactivity. However, the observation of an increase in the apparent molecular weight of both bands in the doublet after spiking with recombinant Smd1 peptide (Fig. 3A) suggests that the doublet represents a modified or partially reduced form of the > 26 kDa protein.

Western blotting revealed that Smd1 defensin was only detected in the reservoir zone homogenates and was not present in other regions of the midgut including the proventriculus, thoracic midgut, opaque zone or lipid zone (data not shown). In addition, Smd1 defensin was not present in the fat body, malpighian tubules, brain or thoracic flight muscle (data not shown).

Purification and N-terminal amino acid sequence

The > 26 kDa protein that complexed with Smd1 was purified by repeated excision of the band from SDS-PAGE and electro-elution until a single band was revealed by SDS-PAGE. The identity of the band was confirmed by Western blotting a sub-sample with anti-Smd1 serum before obtaining the following partial N-terminal amino acid sequence by Edman sequencing: IVGGNAFAHEGQFPHQVSS. Blast searches revealed that amino acids 1–17 of the > 26 kDa protein had 76% identity with amino acids 50–66 of serine protease SP24D from *Anopheles gambiae*, suggesting that this peptide fragment was from a serine protease.

Full length clone sequence and alignment with other serine proteases

Using a degenerate sense primer designed from the least conserved portions of the Edman sequence and the M13–20 primer a full length cDNA was cloned from the library (accession number AY044834). It was 881 nucleotides long, coding for a prepro-protein of 254 amino acids. The predicted molecular weight of the full-length prepro-protein

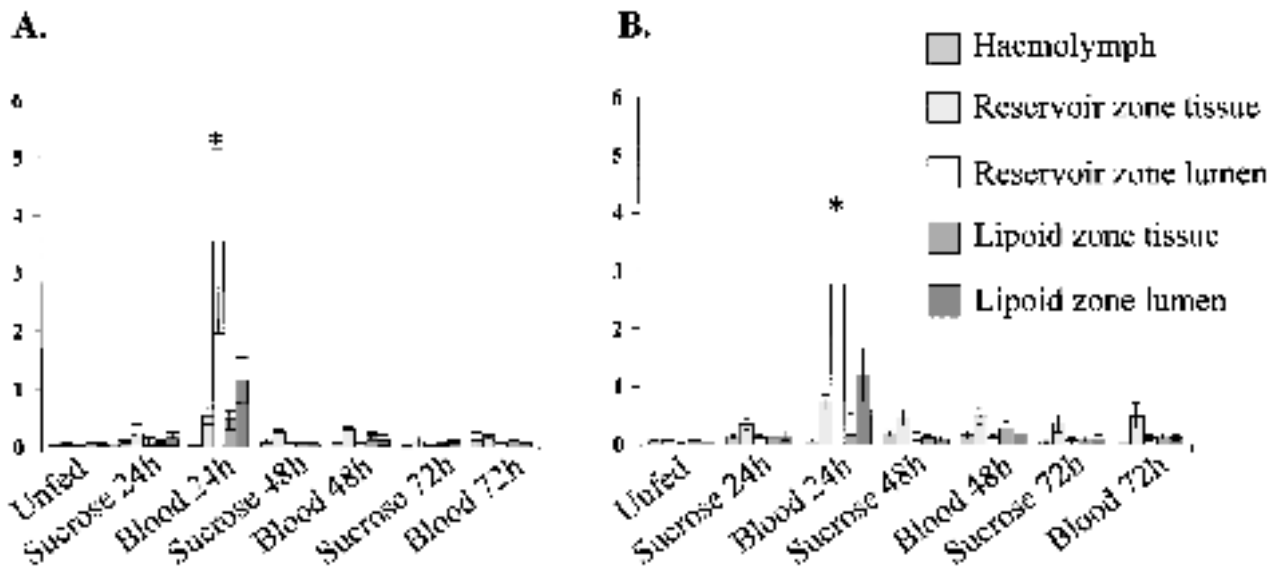


Figure 4. Mean Smd1 and Smd2 levels in adult *Stomoxys calcitrans* haemolymph, reservoir zone tissue, reservoir zone lumen, lipid zone tissue and lipid zone lumen. A, mean and standard errors of Smd1 levels from unfed (UF), sucrose fed (S) and blood fed (B) flies at 24, 48 and 72 h after feeding. Results are from five experiments. B, mean and standard errors of Smd2 levels. * indicates a significant difference from haemolymph, reservoir zone tissue and lipid zone tissue ($P < 0.05$, one-way ANOVA and Fishers pair-wise comparison).

is 27 523.30 Da with a theoretical pI of 5.85 and charge of -4.25 . SignalP analysis V 1.1 (Nielsen *et al.*, 1997) of the sequence suggests that the leader sequence consists of amino acid residues 1–23, which is cleaved between a serine and alanine residue. We suggest the six amino acid activation peptide ARPRPR is cleaved from the mature protein between the arginine and isoleucine at position 30. The theoretical molecular weight, charge and pI of the signal peptide would be 2601.40 Da, $+0.91$ and 8.83, respectively, and for the activation peptide they would be 750.90 Da, $+2.91$ and 12.40, respectively. The mature protein has a theoretical molecular mass of 24 205.00 Da, a charge of -8.25 and a pI of 5.00.

Blastp was used to search GenBank with the > 26 kDa sequence (Altschul *et al.*, 1997) and this revealed homology of the > 26 kDa protein with members of the serine protease S1 family (39% identity with chymotrypsin 1 and 37% identity with serine protease SP24D from *Anopheles gambiae*). Consequently we have named the protein *Stomoxys* serine protease 3 (Ssp3) (accession number AY044834).

Localization of Smd1 and Smd2 peptides and Ssp3 mRNA

The distribution of Smd1 and Smd2 in the adult fly was investigated using ELISA (Fig. 4). Low levels of Smd1 and Smd2 were present in the reservoir zone tissues in unfed and sucrose fed flies. However, in blood fed flies these levels increased at least twofold within 24 h of feeding. The highest levels of Smd1 and Smd2 were detected in the reservoir zone lumen where there was a fortyfold increase in Smd1 and a twenty-threefold increase in Smd2 24 h post blood meal. Gut lumen defensin levels did not increase in

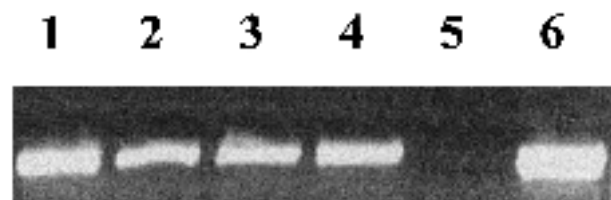


Figure 5. RT-PCR of Ssp3 mRNA. mRNA was prepared from anterior midgut (cardiac, thoracic and reservoir zones, lane 1), posterior midgut (opaque and lipid zones, lane 2), fat body (lane 3) and carcass (lane 4). Negative control (no mRNA, lane 5) and positive control (Ssp3 clone, lane 6).

response to a sucrose meal. This suggests that Smd1 and Smd2 are up-regulated only in response to the blood meal and secreted into the midgut lumen. The finding of defensins in lipid zone lumen presumably reflects passage down the gut lumen from the reservoir because no defensin mRNA is present in lipid zone tissue (Lehane *et al.*, 1997; Munks *et al.*, 2001). Compared to unfed flies the levels of Smd1 and Smd2 in the reservoir tissues, but not the lumen, remain elevated up to 72 h post blood meal. RT-PCR shows that Ssp3 is not restricted to the anterior midgut but is found in all tissues tested in the adult fly (Fig. 5).

Importance of Smd1 and Smd2 in overall midgut antimicrobial response

Zone inhibition assays confirm the presence of antimicrobial activity in midgut homogenates (Fig. 6, zone A). Neither the control antibody nor isotonic saline had any effect on the growth of *M. luteus*. When midgut homogenates were incubated with anti-Smd1 antibody there was a 53% reduction

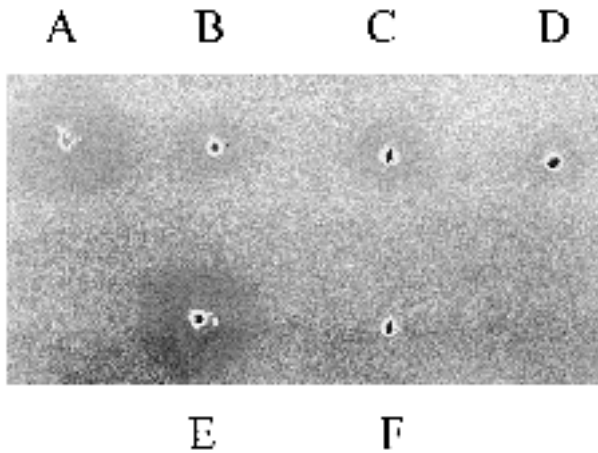


Figure 6. Zone inhibition assay using *M. luteus* as the test organism. Samples in wells were 4 midgut equivalents (w/w protein) from A, gut homogenate alone; B, gut homogenate incubated with Smd1 antibody; C, gut homogenate incubated with Smd2 antibody; D, gut homogenate incubated with both Smd1 and Smd2 antibodies; E, gut homogenate incubated with control antibody; F, Isotonic saline alone.

in the zone of inhibition (Fig. 6, zone B), when homogenates were incubated with anti-Smd2 antibody there was a 47% reduction (Fig. 6, zone C) and when the homogenates were incubated with both antibodies there was a 59% reduction in antimicrobial activity (Fig. 6, zone D) suggesting the presence of other anti-Gram positive agents in the midgut. We were unable to produce recombinant defensins that were exactly the same as the native defensins, therefore we were unable to establish that the concentration of antibodies used in these inhibition assays would be sufficient to inhibit purified defensins. Interestingly, when Smd1 and Smd2 were inhibited by the antibodies *M. luteus* appeared to encroach back into the inhibition zone suggesting that the other antimicrobial agents in the midgut may be bacteriostatic rather than bacteriocidal or that they are relatively unstable.

Discussion

Smd1 and 2 peptides are constitutively produced but ingestion of the blood meal induces up to a fortyfold increase in defensin production in the gut (Fig. 4). The data presented here combined with previous data (Lehane *et al.*, 1997; Munks *et al.*, 2001) suggests that production of these proteins may be regulated post-transcriptionally. Post-transcriptional regulation of genes appears to be a common phenomenon in the midgut of blood-sucking insects (Muller *et al.*, 1995; Lehane *et al.*, 1998; Noriega & Wells, 1999) and may be a consequence of the selective advantage to haematophagous insects of rapid blood meal digestion (Lehane, 1991).

Although we and others have previously demonstrated antimicrobial gene mRNA in insect midgut (Lehane *et al.*,

1997; Dimopoulos *et al.*, 1997; Hao *et al.*, 2001) the data presented (Figs 3C and 4) is the first direct evidence that an antimicrobial peptide is secreted into the insect gut lumen. The massive response to the blood meal contrasted with the weak response to sugar meals (Fig. 4), and the high concentration of defensins in the anterior midgut (where the undigested blood meal is stored) compared to the posterior midgut (where the blood meal is digested), supports the conclusion that midgut defensins help to protect the blood meal from bacterial attack during the 24 h before the meal is fully digested by the fly (Lehane *et al.*, 1997). This conclusion is strengthened by the fact that mRNA for Smd1 and 2 are only found in the anterior midgut (Lehane *et al.*, 1997; Munks *et al.*, 2001) and by the rapid decline in defensin proteins in the gut lumen 24 h post blood meal when the final portions of the stored blood meal have been passed through the gut for digestion. The data presented (Fig. 6) suggests that these two defensins may form only a part, 60% as crudely estimated by zone assays (Fig. 6, zone D), of the anti-Gram positive activity to be found in the anterior midgut. However, we were unable to establish that the concentrations of antibodies used in this assay would fully inhibit purified defensins, therefore these results merely lead us to speculate that there is other anti-Gram positive antimicrobial activity in the anterior midgut.

Our data, particularly the spiking experiments (Fig. 3C), suggest that while in the reservoir tissues the defensins are bound to Ssp3. The presence of a doublet in the spiking experiment (Fig. 3A) may reflect the presence of a modified form of Ssp3. SDS-stable complexes of this sort are well known (Kato *et al.*, 2001). Once the material is secreted into the midgut lumen the two proteins become dissociated (Fig. 3B), which suggests that the Ssp3 defensin aggregate is not the active unit in the midgut lumen. The discrepancy between the apparent molecular weight of Ssp3 at > 26 kDa and the predicted molecular weight of 24.205 kDa may be due to the presence of bound defensin, possible post-translational modifications of the protease, incomplete reduction of the protease during sample preparation or any combination of these possibilities. At present we do not know the mechanism of the interaction(s) between Smd1 defensin peptide and the Ssp3 serine protease. Indeed the number of binding sites available to the defensin on the serine protease and whether the defensin can bind to itself when associated with the protease remains to be elucidated.

The full sequence of mature Ssp3 was used to search GenBank using Blastp (Altschul *et al.*, 1997), which produced multiple alignments with serine proteases, notably insect and vertebrate trypsins and vertebrate elastases. These included interesting examples such as Met-ase-1 (granzyme M) a serine protease from cytolytic granules of rat CD3(-) large granular lymphocytes (Kelly *et al.*, 1996) believed to play a part in innate immune responses in

vertebrates (Sayers *et al.*, 2001). Inspection of these aligned sequences shows Ssp3 contains the catalytic triad, His, Asp and Ser in that order and that the highly conserved regions surrounding the His and Ser residues (which are typical of serine proteases) are also conserved (Kraut, 1977). Ssp3 also has the six highly conserved cysteine residues at the positions that would allow the formation of the three cysteine bonds typical of invertebrate serine proteases and differentiating them from the vertebrate enzymes, which have four such bonds. We conclude that Ssp3 belongs to the peptidase family S1; its particular substrate specificity and function needs to be determined empirically. We note that blood meal digestion does not occur in the reservoir region of the fly where Ssp3 is produced suggesting a non-digestive function for Ssp3. This is supported by both the widespread distribution of Ssp3 mRNA in the body (Fig. 5) and by the relatively poor homology of this serine protease to the two already described digestive serine proteases from *S. calcitrans* (Lehane *et al.*, 1998). It is unlikely that Ssp3 is involved in haemolysis of the blood meal in the reservoir because haemolysis does not occur in this zone (M. J. Lehane, unpublished observation). We have considered the possibility that Ssp3 may play a role in the enzymatic activation of defensin from the pro- to the mature form, which is known to be a key regulatory step in vertebrates (Wilson *et al.*, 1999). It is possible that Ssp3 may be involved in similar activity in the fat body as it is also present in this tissue. Interestingly the cleavage site leading to mature Smd1 is Ala-Ala, which is the preferred site of elastase and the homology searches suggest good homology between Ssp3 and vertebrate elastases. This may be a function of Ssp3. However, enzymatic activation would not require an SDS-stable linkage between the two molecules. A possible function of this tight association may be the inactivation of either the defensin or the trypsin or both while they are within the tissues. Although we are not aware of any other examples of defensin-serine protease associations other SDS-stable complexes have been extensively described for vertebrates. In particular, the interactions of serpins with proteases such as the inhibition of granulocyte proteases by the intracellular serpin, proteinase inhibitor 6 (PI-6) (Scott *et al.*, 1999).

Serine protease has already been immunocytochemically located in *S. calcitrans* reservoir zone secretory granules (Jordao *et al.*, 1996) and it seems probable that the Ssp3 defensin aggregate is localized there. Colocalization of proteases with antibacterial peptides within single secretory vesicles is well documented in vertebrates. For example, it has been shown in mouse Paneth cell granules that defensins (cryptidins) require proteolytic activation by the metalloproteinase matrilysin, which is colocalized in its granules (Wilson *et al.*, 1999). An example of a different type of association is given by the azurophil granule, a specialized lysosome of neutrophils. It contains two families

of antimicrobial proteins, each with four members. The defensins, comprising human neutrophil protein 1, -2, -3 and -4, on the one hand and the serprocidins, comprising cathepsin G, elastase, proteinase 3 and azurocidin, on the other (Gabay & Almeida, 1993). Interestingly antibacterial activity has been reported in an antimicrobial peptide-associated serine protease from the midgut cells of the fly *Sarcophaga peregrina* (Tsuji *et al.*, 1998). The activity of this molecule is an intrinsic characteristic of the protein not related to its protease activity. This protease is found in the yellow body, which is formed from primordial adult midgut cells in the puparium. In a fascinating parallel with our studies it has been found that an antibody to the antibacterial peptide sarcotoxin IA (cecropin family) binds to this 26 kDa protease (Nakajima *et al.*, 1997). The authors suggest either cross reactivity of their antibody with the protease, or that sarcotoxin 1 A forms an SDS-stable complex to the 26 kDa protease. Our data strongly supports the latter hypothesis for the following reason. Smd1 is limited to the reservoir region (Lehane *et al.*, 1997; Munks *et al.*, 2001) while Ssp3 is widely distributed in the tissues of the adult (Fig. 5) and Western analysis shows banding only in the reservoir region where both are present (Fig. 3B). So evidence is accumulating in insects that antibacterial peptides and proteases are colocalized in tissues and, as in vertebrates, that the associated protease may also be antibacterial.

Interactions of insect immune molecules with proteases deserves further study, particularly if immune systems are to be targeted for genetic manipulation in vector-borne disease control.

Experimental procedures

Insects

S. calcitrans was cultured as previously described (Blakemore *et al.*, 1993). The artificial blood meal (Lehane *et al.*, 1998) and sugar meals were made with high purity water (18M Ω).

Production of recombinant defensins

Recombinant proteins were produced using a commercial *Pichia pastoris* system (InVitrogen). The full Smd1 or Smd2 sequence preceded by the sequence defining the KEX2 cleavable segment (Glu-Lys-Arg) of the α -factor mating signal was generated by PCR and inserted into the *Xho*I/SnaB1 or the *Xho*I/*Eco*RI site, respectively, of the plasmid pPIC9. pPIC9 was linearized with *Sal*I and transformed into GS115. *Pichia* was grown in minimum glycerol medium and the inserted gene expressed in minimal methanol medium. The product was purified by HPLC (Lehane *et al.*, 1997) and protein expression levels were determined by the Bradford method (Bio-Rad).

Antibody production

Unique regions of Smd1 and Smd2 were identified by amino acid sequence comparisons and short amino acid sequences (10mers) were commercially synthesized (MWG-Biotech, Germany). The

synthetic peptides were conjugated to bovine thyroglobulin (Sigma-Aldrich, UK) via glutaraldehyde (Adrian, 1997). The primary immunization of female New Zealand White rabbits consisted of 100 nmol of conjugated peptide emulsified with complete Freund's adjuvant (Difco Laboratories, Michigan USA), 1 ml total volume was administered over five subcutaneous (s.c.) sites. Subsequent immunizations consisted of 50 nmol of conjugated peptide emulsified in incomplete Freund's adjuvant and administered over five s.c. sites. Pre-immune serum was collected from each rabbit before the immunization regime commenced, a test bleed was taken from each animal after it had received the primary immunization and two booster injections, which were given at fortnightly intervals.

Each serum was tested by ELISA for reactivity to the synthetic peptide against which it was raised and was subsequently tested against recombinant Smd1 and Smd2 for sensitivity, specificity and cross-reactivity in ELISA and Western blotting. Three sera are used in this study. MJL3, a serum raised against amino acid numbers 1–10 in the mature Smd1 peptide (AAKPMGITCD), MJL8, a serum raised against amino acid sequence numbers 18–27 in the mature Smd2 peptide (AHCLLLGKSG) and MJL10, a control serum from a rabbit immunized with all of the components used for immunizations but without any synthetic peptide.

Haemolymph collection and antigen preparation

Haemolymph was collected from twenty-five adult *S. calcitrans* by puncturing the interthoracic membrane with a 0.33 mm diameter needle attached to a 1 ml syringe (Micro-Fine +, Becton Dickinson, UK) and withdrawing haemolymph from the body cavity taking care to avoid contamination of haemolymph with gut tissue or contents. Saturated phenylthiocarbamide (2 µl) was added to the pooled haemolymph sample to prevent coagulation. Samples were centrifuged at 12 000 *g* for 3 min and the supernatant used for ELISA.

Midgut dissection and antigen preparation

Anterior midguts (proventriculus, thoracic midgut and reservoir), reservoir zones alone, opaque zones alone or lipid zones alone were dissected into 154 mM NaCl (pH 7.2), frozen immediately in liquid nitrogen and stored at –80 °C until required. Groups of twenty-five samples were homogenized in 100 µl of 154 mM NaCl (pH 7.2) and centrifuged at 9000 *g* for 15 min. The supernatant was removed and used in subsequent experiments. Contents of the reservoir zone or lipid zone lumen were collected by removing the midgut region as described above and gently applying pressure along the length of the tissue with a Micro-Fine + needle that had been bent at right angles to the syringe barrel. Care was taken not to damage the gut tissue, the absence of accidental damage was assessed microscopically. The expelled contents were collected and pooled from twenty-five flies then stored at –80 °C until required. Thawed gut lumen samples were used directly in experiments.

Enzyme-linked immunosorbent assay (ELISA)

Microtitre plates (Type M29A, F-form, PS microplates, Dynatech, West Sussex, UK) were coated with 5 µg/ml of the appropriate antigen in carbonate buffer (15 mM sodium carbonate, 35 mM sodium hydrogen carbonate, pH 9.6), overnight at 4 °C. Coating conditions were determined by checkerboard titration. One

hundred microlitre volumes per well of antigen solution, primary and secondary antibodies and substrate were used throughout. The plates were washed three times in PBS + Tween-20 (PBS-Tween; 145 mM sodium chloride, 2 mM sodium dihydrogen orthophosphate, 4 mM disodium hydrogen phosphate, 0.05% Tween-20, pH 7.2) between each step. The plates were blocked with 200 µl/well of 5% skimmed milk powder (Marvel) in PBS-Tween for 2 h at ambient temperature. After washing, the plates were incubated with the appropriate rabbit antisera (diluted 1 : 5000 in PBS-Tween) for 2 h at room temperature. After further washing the plates were incubated with horseradish peroxidase-labelled goat anti-rabbit IgG conjugate (Nordic Immunology, Tilburg, the Netherlands; diluted 1 : 1000 in PBS-Tween) for 2 h at room temperature. Following further washing the extent of binding was measured colourimetrically after the addition of 300 µl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (300 µl of a 20-mg/ml solution diluted in 9.7 ml of 2.3% citric acid solution, pH 4.0) in the presence of hydrogen peroxide (10 µl of 30% v/v H₂O₂). Optical density was measured in an ELISA plate reader (Titertek Twinreader, version 2.01) at wavelength 405 nm (OD_{405nm}). The optical density of each antigen preparation was measured in triplicate for each antibody used.

In order to account for day-to-day and plate-to-plate variability and the different volumes of tissues and/or their contents analysed in this study, two calculations were used. The first was a correction factor that allowed direct comparison between plates, the second was the expression of corrected OD_{405nm} values as units per tissue per fly (UTF).

Correction factor calculation

Each plate contained positive reference peptides (against which the antisera were raised). If the mean optical density of positive reference wells was not exactly 1.0, the correction factor was applied. This correction factor was 1.0/mean optical density of reference positive wells. The mean OD_{405nm} for each antigen preparation was then multiplied by the correction factor obtained for that antibody.

Expression of units per tissue per fly (UTF)

The equation for this was: $xy/5$, where x = volume of sample collected and y = total protein expressed as µg/µl.

The corrected optical density was multiplied by the resulting factor, which was then divided by the number of flies used in the group (typically twenty-five) thus giving UTF. UTFs were also calculated for the control sera and subtracted from the UTFs obtained for anti-Smd1 and anti-Smd2 sera.

Tris-tricine SDS-PAGE and Western blotting

Proteins diluted in sample buffer (2 × sample buffer: 0.1 M Tris, 4% SDS, 5% 2-mercaptoethanol, 0.01% Coomassie blue G250, pH 6.8) and separated in Tris-tricine SDS-PAGE (Schagger & von Jagow, 1987) using a 16.5% T/3% C separating gel, a 10% T/3% C spacer gel and a 4% T/3% C stacking gel. Polypeptide molecular weight markers (Bio-Rad, UK) were simultaneously electrophoresed. After electrophoresis protein samples were stained with Coomassie Blue, silver stain or electrotransferred on to nitrocellulose paper (NCP) for Western blotting. Proteins separated by SDS-PAGE were electrotransferred to NCP (Hybond-C extra, Amersham Life Sciences, UK) according to previously published methods (Towbin *et al.*, 1979). After transfer, the marker

lanes were removed and stained with a colloidal gold solution (Protogold, British BioCell International) according to the manufacturer's instructions. The remainder of the blots were blocked in 5% skimmed milk powder (Marvel) in Tween transblotting solution (TTBS, 20 mM Tris, 0.9% NaCl, 0.1% Tween-20, pH 7.2) for 2 h at ambient temperature. The blots were washed (three times for 5 min) in TTBS, cut into strips and probed with the appropriate rabbit antisera (diluted 1 : 75 in TTBS) for 2 h at room temperature. After subsequent washing (three times for 5 min in TTBS) the blots were incubated in horseradish peroxidase-labelled goat anti-rabbit IgG (diluted 1 : 1000 in TTBS, Nordic Immunology, Tilburg, the Netherlands) for 2 h at room temperature. After further washing the blots were developed by the addition of 4-chloro-1-naphthol (20 mg in 4 ml of methanol) in 16 ml of trans-blotting solution (TBS, 20 mM Tris, 0.9% NaCl, pH 7.2) containing hydrogen peroxide (10 µl of 30% v/v H₂O₂). The reaction was stopped by immersing the blots in distilled water.

Purification of > 26 kDa band and N-terminal sequencing

The > 26 kDa band was purified by preparative tris-tricine SDS-PAGE followed by excision of the correct band and electro elution of the protein of interest. During each PAGE run the ends of the protein band were cut from the gel and Western blots were carried out on each end of the gel with Smd1 antiserum. This enabled us to monitor that we had the correct band and allowed us to align the blotted ends with the gel in order to excise the band of interest. When we had a single band on tris-tricine SDS-PAGE we confirmed its identity by blotting and excised and electro-eluted the protein again. The purified protein was then subjected to tris-tricine SDS-PAGE and electro-transferred on to polyvinylidene fluoride membrane (PVDF membrane, Sigma) in glycine-free CAPS buffer, pH 11.0. The membrane was stained with Coomassie blue and sent for commercial sequencing (Alta Biosciences, University of Birmingham, UK). One amino acid was observed per sequence cycle.

cDNA library, cloning and sequencing

A *S. calcitrans* adult midgut specific cDNA library, estimated to contain 1.4×10^6 individual clones, was constructed in Lambda ZAP (Stratagene) according to the manufacturer's instructions. Eight hundred midguts were used to make the library. The library was plated using *E. coli* XL-1 Blue. A degenerate sense primer was constructed (5'-GGACAATCYCCXAYCA-3') based on the Edman sequence information for the > 26 kDa protein. This degenerate primer and a universal M13-20 primer were then used in PCR with the midgut specific cDNA library to generate a ³²P-labelled probe for screening the library. pBluescript phagemids were excised *in vivo* from the lambda vector using ExAssist helper phage and plated using *E. coli* XL0LR (Stratagene). DNA sequencing was carried out using a Beckman CEQ 2000XL capillary sequencer.

RT-PCR

The primers 5'-CATTGCTACTGGACCAGA-3' and 5'-GGACAATTCCTCACCA-3' were designed from regions of the > 26 kDa protease gene that are not highly conserved in homologous sequences selected by Blastx. Poly A⁺ RNA was extracted from fifteen anterior midguts (cardiac, thoracic and reservoir zones), fifteen posterior midguts (opaque and lipid zones), six fat

bodies and the remains of two carcasses (approximately equal wet weights of tissue) from adult *S. calcitrans* using the Dynabeads system. One tenth of the extract was used in RT-PCR using the Access RT-PCR system (Promega) with one cycle of 45 min at 48 °C, one cycle of 2 min at 94 °C, forty cycles of 30 s at 94 °C, 1 min at 60 °C and 2 min at 68 °C. Finally we performed one cycle for 7 min at 68 °C. Controls omitting reverse transcriptase were used to check for genomic DNA contamination.

Zone inhibition assays

Antibacterial activity was estimated using zone inhibition assays utilizing either *Micrococcus luteus* or *E. coli* D31 (Lehane *et al.*, 1997). Diameters of zones were recorded following 24 h incubation at either 28 °C for *M. luteus* or 37 °C for *E. coli* D31. Midgut homogenates were filtered through a 0.2 µm nylon filter (Whatman) by centrifuging at 25 000 *g* for 15 min to remove any contaminating bacteria that possess antimicrobial activity (e.g. *Serratia marcescens*; J. V. Hamilton, unpublished results). Samples containing 4 midgut equivalents were incubated with either 0.9% saline or antibody (diluted in saline) for 15 min at ambient temperature before loading into the wells of the agarose plate. The plates were incubated at 30 °C for 16–24 h and the zones of inhibition measured.

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