PROTEIN STRUCTURE AND FOLDING:

Epithelial Innate Immunity : A NOVEL ANTIMICROBIAL PEPTIDE WITH ANTIPARASITIC ACTIVITY IN THE BLOOD-SUCKING INSECT STOMOXYS CALCITRANS

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Epithelial Innate Immunity

A NOVEL ANTIMICROBIAL PEPTIDE WITH ANTIPARASITIC ACTIVITY IN THE BLOOD-SUCKING INSECT *STOMOXYS CALCITRANS**

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The gut epithelium is an essential interface in insects that transmit parasites. We investigated the role that local innate immunity might have on vector competence, taking *Stomoxys calcitrans* as a model. *S. calcitrans* is sympatric with tsetse flies, feeds on many of the same vertebrate hosts, and is thus regularly exposed to the trypanosomes that cause African sleeping sickness and nagana. Despite this, *S. calcitrans* is not a cyclical vector of these trypanosomes. Trypanosomes develop exclusively in the lumen of digestive organs, and so epithelial immune mechanisms, and in particular antimicrobial peptides (AMPs), may be the prime determinants of the fate of an infection. To investigate why *S. calcitrans* is not a cyclical vector of trypanosomes, we have looked in its midgut for AMPs with trypanolytic activity. We have identified a new AMP of 42 amino acids, which we named stomoxyn, constitutively expressed and secreted exclusively in the anterior midgut of *S. calcitrans*. It displays an amphipathic helical structure and exhibits a broad activity spectrum affecting the growth of microorganisms. Interestingly, this AMP exhibits trypanolytic activity to *Trypanosoma brucei rhodesiense*. We argue that stomoxyn may help to explain why *S. calcitrans* is not a vector of trypanosomes causing African sleeping sickness and nagana.

Epithelial intestinal innate immunity plays a major role in the control of infectious diseases in vertebrates (1, 2). In invertebrates, data are still fragmentary despite gut epithelium being an essential interface for parasites during their development in insect vectors. Understanding vector biology is a key element in the control of many parasitic diseases. In this context, the comparison of the trypanosome vector *Glossina* with the sympatric but non-vector *Stomoxys calcitrans* is particularly interesting. The tsetse fly *Glossina* spp. is the major vector of the range of trypanosomes that cause African sleeping sickness in humans and nagana in livestock. Stable flies, *Stomoxys* spp., feed on the same vertebrate hosts as tsetse flies and have a very similar digestive physiology and midgut anatomy. Although *Stomoxys* is constantly exposed to trypanosomes, it kills them in the midgut within 2–4 days of ingestion (3). So *Stomoxys* is not a cyclical vector of trypanosomes (although it can act as a mechanical vector, "flying pin"). Why *Stomoxys* is not a cyclical vector of trypanosomes is unknown. Recent studies have shown that the insect immune system plays a determinant role in the fate of trypanosome infections in tsetse flies (4, 5). Consequently, in this report, we address the possibility that the distinction in vectorial capacity between *Glossina* and *Stomoxys* may lie in differences in immune mechanisms.

Trypanosoma vivax matures entirely in the mouthparts of *Glossina*. The other trypanosomes causing nagana and human disease are ingested into the fly midgut where they multiply first in the endoperitrophic space and later in the ectoperitrophic space tightly sandwiched between the peritrophic matrix and the anterior midgut epithelium (6). These trypanosomes then migrate, entirely within the lumenal space of the intestine, to the mouthparts or salivary glands for transmission to a new vertebrate. Consequently it is epithelial rather than systemic immune responses that are likely to have the major bearing on the fate of trypanosome infections. Epithelia constitute the first line of defense in the innate immunity of both vertebrates (reviewed in Ref. 7) and invertebrates (8–10), and AMPs[1] are essential components of this epithelial immunity. For example, the mouse gut epithelium responds to bacterial infection by secretion of α-defensins or cryptdins (reviewed in Refs. 1 and 2), and gut expression of β-defensins is up-regulated by the parasite *Cryptosporidium parvum* (11). In invertebrates, the *S. calcitrans* anterior midgut epithelium responds to microbial infection by post-transcriptional up-regulation of secreted defensins, whereas the mosquito midgut responds to *Plasmodium* infection by up-regulation of a range of immunity factors including several *AMP* genes (reviewed in Ref. 12). We know that some of these AMPs can be active against trypanosomes (4) and other parasites such as *Plasmodium* and filarial worms that also spend part of their life in the gut of a blood-sucking insect (13, 14).

Because of the potential importance of epithelial AMPs for determining vectorial capacity, we have looked for novel AMPs in gut extracts of *S. calcitrans* using biochemical and molecular

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF467987

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[1] The abbreviations used are: AMP, antimicrobial peptide; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; MIC, minimal inhibitory concentration; HPLC, high pressure liquid chromatography; RP-HPLC, reverse phase HPLC; Boc, *t*-butoxycarbonyl; TFE, trifluoroethanol.

approaches. It is already known that the anterior midgut epithelium of *S. calcitrans* produces two defensins (9) that are secreted into the midgut lumen (15, 16). In this study, in addition to these defensins, we identified a novel 42-residue peptide, which we named stomoxyn. Stomoxyn has no sequence homology with other known proteins. This molecule is adult and anterior midgut-specific, where it is constitutively expressed. As shown by a circular dichroism study, stomoxyn adopts an α-helical structure only in the presence of an organic solvent that mimics a phospholipid membrane. Stomoxyn is active against many Gram-negative and Gram-positive bacteria, filamentous fungi, and yeast, and its activity is apparent within a few minutes of exposure. Although stomoxyn has only limited hemolytic activity against bovine red blood cells, it possesses significant trypanolytic effect on trypomastigote forms of *Trypanosoma brucei rhodesiense*. Consequently it may help to explain why *Stomoxys* is not a vector of African trypanosomiasis.

EXPERIMENTAL PROCEDURES

Insect and Tissue Preparation

S. calcitrans were reared as described by O'Brochta *et al.* (17). Adult male and female insects, both of which feed on blood, were used in the experiments. The artificial blood meal (18) and casein hydrolysate meal were made with high purity water. Flies used for HPLC purification of midgut AMPs were fed from cotton wool soaked swabs on either pig blood or casein hydrolysate (20 g/100 ml). At 24–36 h after feeding, anterior midguts (2,000 consisting of proventriculus, thoracic, and reservoir regions) (19) were dissected in 154 mM NaCl and homogenized at 4 °C in 200 mM sodium acetate at pH 4.5. The homogenate was heated to 100 °C for 5 min and centrifuged at 12,000 × *g* for 10 min at 4 °C. Pellet and supernatant were stored separately at −20 °C until used.

Isolation and Structural Characterization of AMPs

AMP Purification—The supernatant of *S. calcitrans* midgut homogenate was subjected to RP-HPLC on an Aquapore OD300 C_{18} column (220 × 4.6 mm, Brownlee™) with a linear gradient of 2–60% acetonitrile in acidified water (0.05% trifluoroacetic acid) over 120 min at a flow rate of 0.8 ml/min at 35 °C. The column effluent was monitored by absorbance at 225 nm, and fractions were hand-collected. After evaporation under vacuum (Speedvac, Savant), fractions were reconstituted in MilliQ™ water (Millipore). An equivalent of 40 midguts/2 μl was tested for antimicrobial activity using a solid growth inhibition zone assay. HPLC fractions with antimicrobial activity were further purified to homogeneity on a microbore Aquapore RP 300 C_8 column (1 × 100 mm, Brownlee™) by appropriate linear biphasic gradients of acetonitrile in 0.05% trifluoroacetic acid over 60 min at a flow rate of 80 μl/min. The column effluent was monitored by absorbance at 214 nm. Purity of the fractions with antimicrobial activity was controlled between each chromatography by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Pure peptides were finally subjected to Edman degradation. All HPLC purifications were performed at 35 °C with a Waters HPLC system equipped with a pump model 626, a controller model 600S, and a UV detector model 486.

MALDI-TOF MS—MALDI-TOF MS analysis was performed on a Bruker Daltonique (Bremen, Germany) BIFLEX III™ mass spectrometer in a positive linear mode with an external calibration. Samples were prepared according to the sandwich method (20) following the procedure reported previously (21).

Microsequence Analysis—Automated Edman degradation of the purified AMPs and detection of the phenylthiohydantoin derivatives were carried out using pulse liquid automatic sequencer (Applied Biosystems Inc., model 473A).

Library Construction, Screening, and Sequencing

A *S. calcitrans* adult midgut cDNA library with an estimated complexity of 1.4×10^6 plaque-forming units was constructed in Lambda ZAP (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The library was plated using *Escherichia coli* XL-1 Blue. A degenerate sense PCR primer (S1 5′-ATGACRGCNGTRTCYTT-3′) corresponding to the amino acid sequence KDTAVI of stomoxyn and an M13−20 primer were used in PCR with the cDNA library as template to generate a 336-bp product. This product was cloned using TOPO TA (Invitrogen) and sequenced. A pair of sequence-specific primers were designed from this sequence (S2, 5′-TGTATCCAATTCACTTTTCA-3′, and S3, 5′-TATGACAGCAGTATCCTT-3′) and used in PCR with the cDNA library as template to generate a 193-bp probe. The probe was ^{32}P-labeled and used to screen the midgut cDNA library. Phagemid clones (pBluescript) containing the stomoxyn protein cDNA were excised *in vitro* from the λ vector using ExAssist helper phage and plated using *E. coli* XLOLR (Stratagene) and sequenced.

Nuclease Protection Assays

Nuclease protection assays were performed using the Multi-NPA kit (Ambion, Austin, TX). Total RNA was prepared from 10 anterior midguts (proventriculus, thoracic, and reservoir regions), 10 posterior midguts (opaque and lipoid regions), 1 remains of a carcass from adult *S. calcitrans*, 100 eggs, and 2 larvae or 2 pupae using RNeasy Mini Kit (Qiagen, Crawley, UK). For treatment details, see Fig. 3 capture. Oligonucleotide probes NPA5 (5′-GCCTCGGTCTGGGTGGCCGAAAGGCATAGCACCAAAACGACCAGAACTACCAGGAATCCACTAA-3′ and NPA4 5′-GCGTGTGGAATCGGAAGGAATGGGAGTAACATCTTCTTATAGAT-3′) were used to detect stomoxyn and S14, respectively. Each oligonucleotide probe contained 10 bp of non-complementary sequences on the 3′ end so that full-length probe can be distinguished from the protected fragment.

Peptide Synthesis

The peptide was assembled using a multichannel peptide synthesizer adapted to Boc chemistry (22). Classical Boc-protected amino acids were used during the assembly but with histidine residues introduced as Boc-His(dinitrophenyl) derivatives. Following the last Boc deprotection, the resin was washed five times with dichloromethane and dried under nitrogen. Finally a standard fluorhydric acid procedure was used for deprotection and cleavage of the peptide from the resin. After removal of the fluorhydric acid by vacuum, the resin was washed with ethyl ether, and the peptide was extracted with acetic acid, water, and acetonitrile. The synthetic peptide was purified to homogeneity by solid-phase extraction and RP-HPLC using a gradient of acetonitrile in acidified water. Peptide purity and integrity were controlled by MALDI-TOF MS.

Bioassays

Solid Growth Inhibition Zone Assay—During the peptide purification, antimicrobial activity was assayed against the two test bacteria, the Gram-positive *Micrococcus luteus* (23) and the Gram-negative *E. coli* K12 RM148 (9). Briefly, 2 μl (40–230 midguts, depending on the purification step) of each RP-HPLC fractions were incubated with bacteria overnight at 37 °C. Two AMPs were used as positive controls: *Drosophila* cecropin A (25 μM) and *Aedes* defensin A (15 μM) for anti-Gram-negative and -positive activities, respectively.

Liquid Growth Inhibition Assay—The activity spectrum (minimal inhibitory concentration, MIC) of stomoxyn (concentration range, from 0.2 up to 100 μM) was determined against bacteria and fungi using a liquid growth inhibition assay (23). The strains used were from private and public collections (24, 25). Bactericidal effect was measured by colony-forming unit counting at 24 h. When the antimicrobial activity was assayed in the presence of salts, the appropriate medium was supplemented with 1 mM $CaCl_2$, 50 mM KCl, or 154 mM NaCl.

Kinetic of Killing against Bacteria and Yeast—Overnight cultures of *E. coli* K12 RM148, *M. luteus*, and *Cryptococcus neoformans* were diluted in appropriate media and allowed to grow to logarithmic phase. Stomoxyn (10 times the MIC) was incubated with the test microorganism in a 96-well microtiter plate at 37 °C. At different time points (5, 15, 45, and 150 min), samples were removed, diluted, and plated on Luria Bertani broth or Sabouraud agar plate for colony-forming unit counting.

Kinetic of Killing against Filamentous Fungi—Fungal spores (10^4/ml) of *Neurospora crassa* were incubated in Eppendorf tubes with 25 μM stomoxyn (4 times the MIC value). At different time points (30, 60, 150, 270 min, and overnight), spores were washed twice with fresh potato dextrose broth (half-strength) and incubated for an additional 48 h. Fungal growth was measured at 550 nm.

Antitrypanosomal in Vitro Assay—The antitrypanosomal assay was performed using the Alamar Blue assay according to Raez *et al.* (26). Briefly, bloodstream forms of *T. b. rhodesiense* STIB 900 were added to sterile 96-well microtiter plates in 100 μl of culture medium according to Baltz *et al.* (27) containing 300 bloodstream forms with or without a 3-fold serial drug dilution. The top concentration of stomoxyn was 500 μg/ml (approx. 113 μM). The test was run in duplicate. After 72 h of incubation, 10 μl of Alamar Blue were added to each well, and the

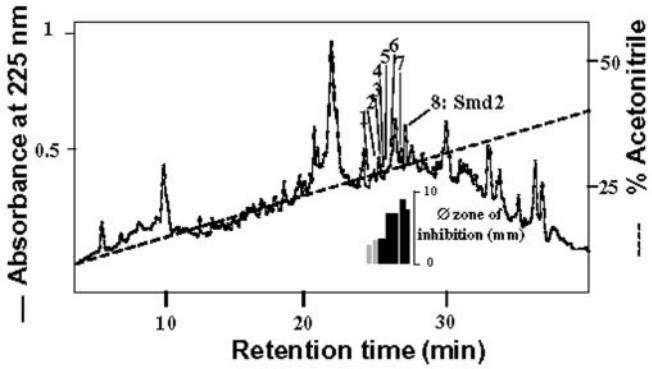

FIG. 1. **RP-HPLC of *S. calcitrans* adult gut tissue.** The antimicrobial activity, detected using a solid growth inhibition zone assay, is expressed in diameter (mm). Fractions (*numbered*) active against *M. luteus* are in *gray boxes*, and fractions active against *E. coli* are in *black boxes*. Elution was performed using a gradient of acetonitrile (*dotted line*), and the absorbance was measured at 225 nm (full line). Smd2 corresponds to the *Stomoxys* midgut defensin 2 identified previously (9).

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aaatgaatttctacaaatacctggtagttctggtcgtttt 40
   M  N  F  Y  K  Y  L  V  V  L  V  V  L 80
ggtgctatgcctttcggccacccagaccgaggcaagaggt
  V  L  C  L  S  A  T  Q  T  E  A  R  G 120
tttcgcaaacacttcaataaattggtgaaaaaagtcaag
 F  R  K  H  F  N  K  L  V  K  K  V  K  158
cacaccatttcggaaacagctcatgtggccaaggatact
 H  T  I  S  E  T  A  H  V  A  K  D  T  198
gctgttatagctggaagtggagctgctgtagttgctgcc
 A  V  I  A  G  S  G  A  A  V  V  A  A  237
actggttaaaacagaagcctggaattatgttgaaaaacat
 T  G                                    277
aaaatgatcatttttcaaaaaaaaaaaaaaaaaaaaa
```

FIG. 2. **The nucleotide sequence of stomoxyn cDNA and the deduced amino acid sequence.** The mature peptide coding sequence is shown in *bold* with amino acid residues *below*. Untranslated 5′ and 3′ sequences are shown in *italics*, and the start and stop codons are *underlined*. The *dotted line* marks the glycine residue involved in the C-terminal amidation of the threonine residue.

plates were incubated for another 2–4 h. The plates were read in a microplate fluorometer system (Spectramax Gemini, Molecular Devices) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were transferred into a graphic program (Softmax Pro, Molecular Devices), sigmoidal inhibition curves were determined, and IC_{50} (drug concentration inhibiting 50% of fluorescence development) values were calculated. The activity against procyclic forms was determined in the same way. Procyclics of the same trypanosome strain were grown in SDM-79 medium (28).

Hemolytic Assay—Bovine red blood cells were washed twice with phosphate buffer saline by centrifugation at 3,000 rpm, 10 min at 4 °C until the supernatant was clear. A 10% suspension of bovine red blood cells was incubated in Eppendorf tubes in the presence of 2-fold dilutions of stomoxyn (0.2–100 μM) for 1 h at 37 °C. Samples (100 μl) were centrifuged at 3,000 rpm for 5 min, and 90 μl of supernatant were transferred in a microtiter plate. The absorbance of the supernatant was measured at 405 nm. As a positive control (100% lysis), bovine red blood cells were incubated with 0.1% SDS.

Serum and Trypsin Stability—The stability of stomoxyn (12 μM final concentration) in the presence of 25% bovine serum was tested according to the procedure described previously (29) except that the stability was evaluated by MALDI-TOF MS. For the trypsin stability study, stomoxyn (0.5 mM in a final volume of 20 μl) was incubated with bovine trypsin (Roche Molecular Biochemicals) at an enzyme/peptide ratio of 1/10, according to the manufacturer's directions. At different time points (5, 15, 30, 60, 150 min, and overnight), an aliquot (0.5 μl) of the sample was removed, and the reaction was stopped with trifluoroacetic acid. The digest products were analyzed by MALDI-TOF MS.

Circular Dichroism Analysis—Circular dichroism spectra of stomoxyn (0.1 mM) were recorded between 180 and 250 nm on a Jasco J-810 spectropolarimeter in 0.5 nm steps at varying concentrations of trifluoroethanol (TFE) in water (from 0 to 80%, v/v) at pH 4 using a quartz cell of 1 mm. For each experiment, four spectra were averaged, and the baseline was corrected for neat solvent and solvent mixture. All measurements were carried out at 25 and 4 °C.

RESULTS

Stomoxyn Peptide Purification and Characterization

Crude homogenates of anterior midgut of *S. calcitrans*, 24–36 h after a blood meal, have been shown previously to possess anti-Gram-negative activity. Two insect defensins have already been identified (Smd1 and Smd2 (9)). In this study, screening of HPLC-purified proteins extracted from 2,000 anterior midguts revealed the presence of eight fractions with antimicrobial activities. Two fractions were active against the Gram-positive test organism *M. luteus* (Fig. 1, *peaks 1* and *2*), and six were active against the Gram-negative strain *E. coli* K12 RM148 (Fig. 1, *peaks 3–8*). After MALDI-TOF MS measurement and partial Edman sequencing, fraction 8 was found to contain *Stomoxys* defensin Smd2 (data not shown). As the strongest activities were observed in fractions 5 and 7 (Fig. 1), an extensive purification was performed on these fractions, and pure active compounds with molecular masses of 4246.11 MH^+ and 4415.49 MH^+ were measured for fractions 5 and 7, respectively (data not shown). For the molecule at 4246.11 MH^+, the following 33-amino acid partial sequence was obtained, RGFRKHFNKLVKKVKHTISETAHVAKDTAVIAG, whereas for the molecule at 4415.49 MH^+, a 30-amino acid partial sequence was obtained that was identical to the previous one. As searches in FASTA.GENOME and the SwissProt data base using the Blosum 50 scoring matrix did not establish significant similarity with already known peptides/proteins, we named this new molecule stomoxyn. The full-length stomoxyn sequence was obtained by screening of a midgut cDNA library. The sequence of the stomoxyn cDNA clone is 314 nucleotides long including 42 nucleotides of 5′-untranslated and 68 nucleotides of 3′-untranslated sequence flanking an open reading frame corresponding to 67 amino acid residues of the precursor protein (Fig. 2). According to the results of Edman degradation and cDNA cloning, the calculated molecular mass of mature stomoxyn is 4474.22 MH^+. The mass difference observed between this calculated mass and the mass measured for the active compound differed from 59 Da, corresponding to the lack of the C-terminal glycine residue and to amidation of the C-terminal threonine residue.

Expression of Stomoxyn mRNA in S. calcitrans

The expression of stomoxyn mRNA in different tissues and at various developmental stages in *S. calcitrans* was determined by nuclease protection assays (Fig. 3). Stomoxyn is expressed in the anterior midgut (proventriculus, thoracic, and reservoir regions) but not in the posterior midgut (opaque and lipoid regions) or the rest of the adult body, eggs, larvae, or pupae of *S. calcitrans*. The level of cDNA appeared to be unchanged with blood feeding, with and without addition of bacteria and laminarin. However, it should be noted that other immune peptides in these tissues are strongly regulated at the post-transcriptional level (16).

Activity Spectrum of Stomoxyn

Antimicrobial Activity—To investigate the antimicrobial properties of stomoxyn, the peptide was synthesized using Boc chemistry, purified to homogeneity, and tested against various microorganisms (Gram-negative and Gram-positive bacteria, filamentous fungi, and yeasts). The activity of stomoxyn was compared with a standard α-helical AMP from insects, *Drosophila* cecropin A. Stomoxyn had marked activity against

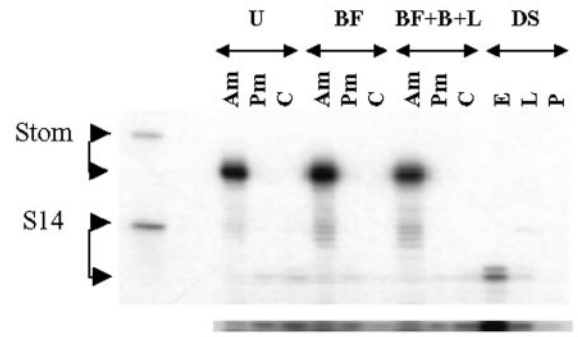

FIG. 3. **Expression of stomoxyn mRNA by nuclease protection assay.** The expression of stomoxyn was measured in anterior midgut (*Am*) (proventriculus, thoracic, and reservoir regions), posterior midgut (*Pm*) (opaque and lipoid regions), and remainder of carcass (*C*) of unfed flies (*U*), artificial blood-fed flies (*BF*), or artificial blood supplemented with *M. luteus* and *E. coli* K12 RM148 and laminarin-fed flies (*BF+B+L*). Different developmental stages (*DS*) were also tested: eggs (*E*), larvae (*L*), or pupae (*P*). The S14 ribosomal gene (constitutively expressed) was used as loading standard.

TABLE I
Antimicrobial activity spectrum of stomoxyn (Stom) and Drosophila cecropin A (Cec)

The antimicrobial activity spectrum was established in liquid growth inhibition assays. Two-fold serial dilutions were tested from 100 μM down to 0.19 μM. The activity is reported as MIC values in μM. ND means No Data, and NA means no activity detected at the highest concentration tested (100 μM).

Measurement Microorganisms	MIC	
	Stom	Cec
	μM	
Gram negative bacteria		
E. coli K12 RM148	0.19–0.39	ND
E. coli D22	0.19–0.39	0.1–0.25
E. coli D31	0.19–0.39	0.1–0.25
E. coli SBS363	< 0.19	0.19–0.39
E. coli 1106	0.19–0.39	0.25–0.5
Enterobacter cloacae β12	3.12–6.25	0.78–1.56
Erwinia carotovora	0.78–1.56	0.78–1.56
Klebsiella pneumoniae	0.78–1.56	0.39–0.78
Pseudomonas aeruginosa	0.39–0.78	1–2.5
Salmonella typhimurium	0.39–0.78	0.5–1
S. marcescens Db 11	NA	NA
Xanthomonas campestris	0.39–0.78	0.5–1
Gram-positive bacteria		
Aerococcus viridans	0.78–1.56	5–10
Bacillus cereus	NA	NA
Bacillus megaterium	0.78–1.56	1.56–3.12
B. subtilis	6.25–12.5	10–25
Enterococcus faecalis	0.78–1.56	NA
L. monocytogenes	NA	NA
M. luteus	0.78–1.56	5–10
S. aureus	NA	NA
Streptococcus pyogenes	1.56–3.12	NA
Filamentous fungi		
A. fumigatus	50–100	NA
B. bassiana	NA	NA
Fusarium culmorum	0.39–0.78	1–2.5
Fusarium oxysporum	0.78–1.56	1–2.5
Nectria haematococca	0.39–0.78	ND
N. crassa	3.12–6.25	2.5–5
Trichoderma viride	1.56–3.12	ND
Trichophyton mentagrophytes	3.12–6.25	ND
Yeast		
C. albicans	25–50	NA
Candida glabrata	25–50	NA
C. neoformans	0.78–1.56	NA
S. cerevisiae	NA	NA

most of the microorganisms tested (Table I). All the Gram-negative bacteria tested, except *Enterobacter cloacae* (6 μM), were affected by less than 2 μM peptide. No activity could be

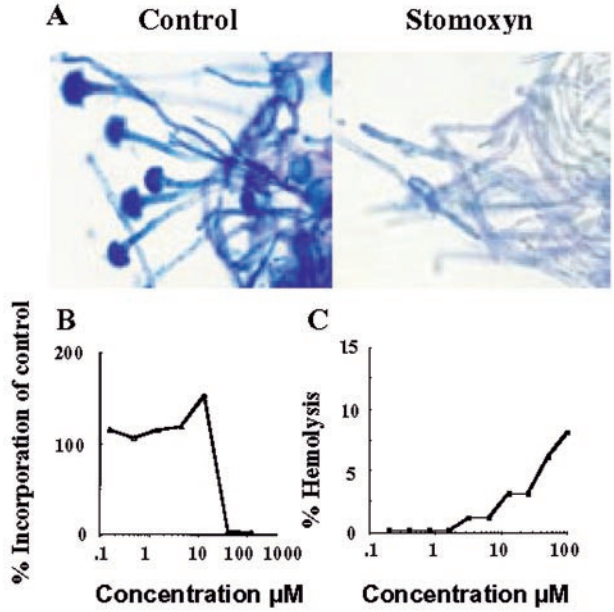

FIG. 4. **Biological activity of stomoxyn against a filamentous fungus, parasites, and red blood cells.** *A*, inhibition of *A. fumigatus* sporulation. Light microscopy observation of *A. fumigatus* after 48 h of incubation with stomoxyn (25 μM) or with water as control is shown. The preparation was stained with aniline blue to increase contrast. *B*, effect of stomoxyn on trypanosomes. The peptide was incubated at different concentrations (3-fold serial dilutions with trypomastigote forms of *T. b. rhodesiense*). After 72 h, the trypanolytic effect was measured by the Alamar blue technique. *C*, hemolytic activity on bovine red blood cells. 2-fold serial dilutions of stomoxyn were assayed against a 10% solution of red blood cells for 1 h at 37 °C. After centrifugation, the absorbance of the supernatant was measured at 405 nm.

detected against the entomopathogenic Gram-negative bacteria, *Serratia marcescens*, up to 100 μM. These activities are comparable with *Drosophila* cecropin A. Synthetic stomoxyn also showed high activity against most of the Gram-positive bacteria tested (MICs < 3 μM), except for *Bacillus subtilis* (MIC = 6.25–12.5 μM). No activity was detected against *Bacillus cereus*, *Listeria monocytogenes*, or *Staphylococcus aureus* up to 100 μM peptide. For all the Gram-positive bacteria tested, stomoxyn was more active than *Drosophila* cecropin A. In addition, stomoxyn was bactericidal on all the bacterial strains affected (data not shown). Most of the filamentous fungi assayed were highly sensitive to stomoxyn (MIC < 6.25 μM), whereas only a few strains were affected by *Drosophila* cecropin A. The human pathogenic strain *Aspergillus fumigatus* was only moderately affected by 100 μM stomoxyn, and *Beauveria bassiana* was unaffected, whatever the doses. However, *A. fumigatus* sporulation was inhibited by less than 6 μM stomoxyn (Fig. 4*A*). In contrast to *Drosophila* cecropin A, which was inefficient on the four yeast strains tested, stomoxyn was highly active against *C. neoformans* (MIC = 0.78–1.56 μM), moderately effective against *Candida albicans* and *C. glabrata* (MIC = 25–50 μM), and inefficient against *Saccharomyces cerevisiae* up to 100 μM (Table I). Killing assays on the different pathogens (bacteria, filamentous fungi, and yeast) revealed that stomoxyn acted within the first few minutes of incubation (data not shown). The antimicrobial activity of stomoxyn was not affected by various concentrations of different salts such as KCl, $CaCl_2$, or NaCl (data not shown).

Activity of Stomoxyn against Eukaryotic Cells—The antiparasitic activity of stomoxyn was tested against trypomastigote (vertebrate host forms) and procyclic (insect forms) forms of *T. b. rhodesiense*. The peptide was found to be lytic at 113 μM with an inhibitory concentration (IC_{50}) of 37 μM on trypomas-

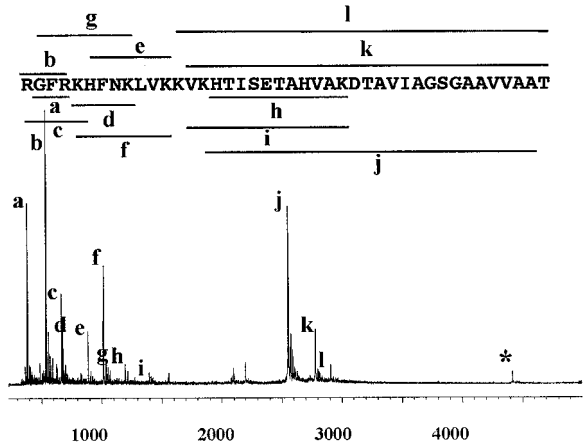

FIG. 5. **Stability of stomoxyn to trypsin.** MALDI-TOF mass spectrum of the digest after 5 min of incubation at 37 °C of stomoxyn (50 μg) with trypsin (5 μg). The labeled peaks (*a–l*) point out the identified tryptic fragments of stomoxyn. The labeled *full lines above* and *below* the primary structure of stomoxyn indicate the corresponding amino acid sequences. The *asterisk* stands for native stomoxyn.

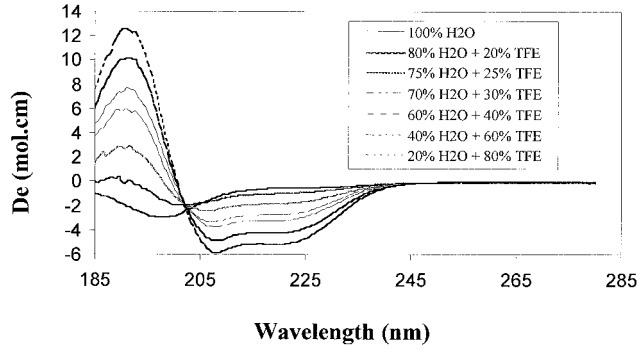

FIG. 6. **Circular dichroism spectra of synthetic stomoxyn at 25 °C and at varying concentrations of TFE.**

tigote bloodstream forms (Fig. 4*B*). Against procyclic *T. b. rhodesiense*, the peptide was found to be inactive at the highest concentration tested (113 μM; data not shown). Finally, as the anterior part of the midgut is a major site for blood storage and dehydration (30) but not hemolysis (31), stomoxyn was tested for its hemolytic activity. At 10 μM, stomoxyn exhibited hemolytic activity of $< 4\%$ on bovine red blood cells, whereas at 100 μM, hemolytic activity did not exceed 10% (Fig. 4*C*).

Stomoxyn Stability in Bovine Serum and to Trypsin

Because stomoxyn is present in the digestive tract where the blood meal is stored, we evaluated the stomoxyn resistance to bovine serum. To retard the kinetic of degradation and to increase peptide recovery, the stability experiment was performed in diluted serum (25%). After incubation of stomoxyn with bovine serum for 45 min at 37 °C, MALDI-TOF MS did not reveal any significant cleavage product or reduction of the initial amount of native peptide (data not shown). However, after a 2-h incubation, a strong decrease in the amount of stomoxyn was observed through estimation based on the MALDI-TOF MS peak heights. When evaluating the first metabolites at 2 h, only C-terminal fragments of stomoxyn can be detected by MALDI-TOF MS, and no clear signal corresponding to the N-terminal part was detected. After 4–5 h of incubation, no stomoxyn could be detected.

To assay the susceptibility of stomoxyn to trypsin, an enzyme present in abundance in the posterior midgut of the stable fly, a high concentration of peptide (0.5 mM) was incubated with pure trypsin, and the digest products were analyzed by MALDI-TOF MS in a time course experiment. After 5 min of incubation at 37 °C, only traces of native stomoxyn were visible (Fig. 5). Precise mass spectrometric analyses of the crude digest revealed 12 tryptic fragments in the mass range of 400 Da to 3 kDa covering all the primary structure of stomoxyn (Fig. 5), confirming the extreme sensitivity of this peptide to trypsin.

Circular Dichroism Spectroscopy

The circular dichroism spectra of stomoxyn were acquired in increasing concentrations of TFE from 0 up to 80% at 4 and 25 °C. In the absence of TFE and at 20% TFE, stomoxyn spectra are characteristic of a random coil conformation. As the TFE concentration increased, a shoulder at 222 nm and a shallow minimum in the vicinity of 208 nm appeared, indicating a transition from a flexible structure to a helical conformation of the peptide in the presence of TFE. A marked effect of the temperature was visible at 30 and 40% TFE. At an equivalent concentration of TFE, the helicity was lower at 25 °C (Fig. 6) than at 4 °C (data not shown).

DISCUSSION

The present study reports the identification of stomoxyn, an AMP in the stable fly *S. calcitrans* with no homology with known proteins. Stomoxyn has no cysteine residue and adopts an α-helical structure in an organic solvent mimicking the phospholipidic membranes. As deduced from cDNA cloning, Edman degradation, and mass spectrometry measurement, stomoxyn is post-translationally modified by C-terminal amidation of the threonine residue. A truncated form of stomoxyn (molecule at 4256.11 MH^+) lacking the two last C-terminal residues (Ala-Thr) was also isolated, probably corresponding to degradation by exopeptidases. We do not know whether this results from a natural degradation within the gut or from our experimental conditions. Alignment of the prepropeptide sequence of *S. calcitrans* stomoxyn and data base sequences revealed very little identity with any known peptide. The closest identity ($p = 0.44$) was with a cecropin 3 precursor molecule (swiss protein database accession number Q94558) derived from *Drosophila virilis*, although little identity was found in the mature peptide. The greatest identity was between the first 5 amino acids of the prepropeptide domain of these molecules with the identical sequence, MNFYK. A second region of identity was found in the prepropeptide sequence KYLVVLVVLVLCL, which showed some similarity ($p = 1.9$) to the andropin precursor (accession number P21663) derived from *Drosophila melanogaster* (32).

As shown by nuclease protection assays, stomoxyn is specifically expressed in the anterior part of the midgut where the blood is stored. Blood is a highly valuable resource, which the fly must protect from microorganisms during storage prior to digestion. It is likely that stomoxyn, with its rapid acting broad range of activity against both bacteria and fungi (Table I), plays an important role in this protection. In previous studies on gut immunity of *S. calcitrans*, two defensins, Smd1 and Smd2, have been described that are also specific to the anterior midgut of adult flies (9, 15). This production of at least three AMPs that are specific to adult midgut tissues illustrates the importance of the gut in the innate immunity of this blood-sucking insect and opens the possibility for the synergistic action of these various AMPs. Using the synthetic stomoxyn as reference, the quantity of native stomoxyn was estimated by μ-RP-HPLC to be ~530 pg/gut. We were not able to quantify the local concentration of stomoxyn. However, given that stomoxyn is likely to be secreted into the very confined space between the gel-like, dehydrated blood meal and the midgut epithelium, we speculate that the local concentration can reach a locally high

level as observed for vertebrate defensins (2). The synthesis of AMPs in the anterior part of the gut seems to be a conserved phenomenon in insects. Gambicin, cecropin, and defensin in *Anopheles gambiae* (8, 21, 24) and diptericin in *D. melanogaster* (10) are also secreted in the anterior part of the midgut. Digestive enzymes, secreted in the posterior part of the gut, may inactivate AMPs, as shown by the rapid degradation of stomoxyn by trypsin (Fig. 5).

Stomoxys is sympatric with *Glossina*, feeds on many of the same vertebrate hosts, and is thus regularly infected with trypanosomes but kills them efficiently in the gut (3). *Glossina* only feeds on vertebrate blood, whereas *Stomoxys* will feed on a variety of other materials in addition to blood including various sugar sources such as rotting fruit. As a consequence, the midgut of *Stomoxys* almost certainly receives a much stronger and more regular microbial challenge than that of the tsetse fly. So we might reasonably predict that the epithelial immune system of *S. calcitrans* is more highly developed than that found in the tsetse fly. The picture emerging to date is that the midgut of *Glossina* secretes defensin and diptericin using genes, which are also expressed in fat body (4). In contrast, *S. calcitrans* has at least three immune genes, which are specialized for exclusive use in the anterior midgut epithelium, two defensins (9) and stomoxyn. To control for the presence of the stomoxyn peptide in the tsetse genome, we carried out PCR experiments using two sets of stomoxyn primers and *G. morsitans morsitans* genomic DNA as template. Although stomoxyn was present in the *S. calcitrans* midgut cDNA library used, no such gene has been found in either *G. m. morsitans* genomic DNA or a *G. m. morsitans* midgut cDNA library (data not shown). In addition, stomoxyn shows an exceptionally wide spectrum of activities including trypanolytic activity on trypomastigote bloodstream forms of *T. b. brucei*. The fact that stomoxyn exhibited no antitrypanosomal effect against the procyclic stage indicates that the activity on the bloodstream forms must be fast before the first procyclic forms appear in the midgut, 24–48 h after ingestion of the infected blood meal. Although trypanolysis occurs at a relatively high concentration on trypomastigote forms, such concentrations may well occur *in vivo* for the following reason. When a blood meal is taken, it strongly distends the peritrophic matrix, pushing it hard against the midgut epithelium. It is then rapidly dehydrated, giving the blood meal a gel-like consistency. Secretion of AMPs at this time is likely to lead to high local concentrations of AMPs in the very confined space between the anterior midgut epithelium and the gel-like blood meal, distending the peritrophic matrix. Interestingly, it is in this confined space that the developing trypanosomes concentrate. It is interesting to note that when α-defensins are secreted into the restricted space of the cryptal well in the vertebrate gut, local concentrations as high as a grams per liter range are estimated to occur (2). We suggest that the presence of stomoxyn in the anterior midgut of *S. calcitrans* may help to explain why *Stomoxys* is not a biological vector of trypanosomes.

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