Molecular cloning of the trypsin inhibitor from the skin secretion of the Madagascan Tomato Frog, Dyscophus guineti *(Microhylidae), and insights into its potential defensive role*

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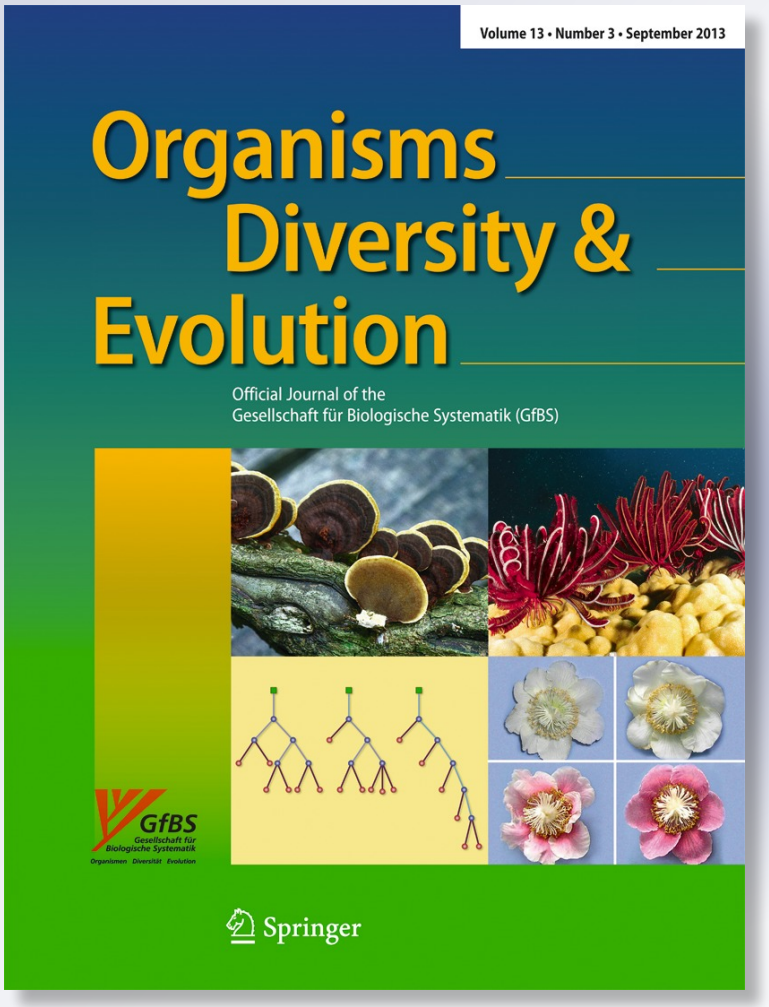

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METHODS AND APPLICATIONS

ORGANISMS DIVERSITY & EVOLUTION

Molecular cloning of the trypsin inhibitor from the skin secretion of the Madagascan Tomato Frog, *Dyscophus guineti* (Microhylidae), and insights into its potential defensive role

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Abstract In this study, we investigate the skin secretion of the Madagascan Tomato Frog, *Dyscophus guineti*, which is characterized by its peculiarly adhesive and viscous nature, with a view toward the function of the member of the Kunitz/bovine pancreatic trypsin inhibitor family (BPTI) it is known to contain. Using "shotgun" cloning of a skin secretion-derived cDNA library, we obtained the full-length sequence of the respective precursor that encodes this trypsin inhibitor. Furthermore, we demonstrated that this enzyme has inhibitory activity against trypsin, but not against thrombin, and also has no antimicrobial activity. Moreover, we confirm that it appears to be the only bioactive peptide in the skin secretion of this species. Using these observations, we attempt to posit a role for this inhibitor. In particular, we hypothesize that the trypsin inhibitor in *D. guineti* (and possibly other microhylid frogs) maintains the soluble state of the skin secretion during storage in the glands. Upon discharge of the secretion, the trypsin inhibitor, which occurs in low concentrations, can no longer prevent the polymerisation process of other yet unidentified skin proteins, thereby resulting in the conversion of the secretion to its final glue-like state. Thus, the major defensive value of the skin secretion appears to be mechanical, impeding ingestion through a combination of adhesion and the body inflation typical for some microhylid frogs rather than chemical through antimicrobial activity or toxicity.

Keywords Amphibian skin · Host-defence · Protease inhibitors · Adhesive secretion · cDNA

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Introduction

Amphibians possess a highly specialised skin that fulfils a large number of important physiological functions, including respiration, water balance, and defence. The latter function is achieved through the production of a vast quantity of different molecules such as alkaloids, steroids (bufadienolides), biogenic amines, and biologically active peptides (Bevins and Zasloff 1990; Daly et al. 2005; Erspamer 1994; Steyn and van Heerden 1998), all of which are stored in the cutaneous granular (poison) glands. Of these different compounds, peptides are by far the most diverse class contributing to host defence in frogs and toads (Anura) and have been isolated from a wide range of species (Pukala et al. 2006; Bevins and Zasloff 1990). They are typically classified according to their different biological activities and include tachykinins, bradykinin-related peptides, caeruleins, bombesins, tryptophyllins, various families

with antimicrobial activity (together, antimicrobial peptides or AMPs), and protease inhibitors. In this article, our focus lies with the latter two classes of biologically active peptides.

Protease inhibitors are ubiquitously present across all organisms, ranging from prokaryotic microorganisms to plants and animals, and are typically classified according to the reactive amino acid residue in the catalytic site responsible for the specific mechanism of proteolytic action (e.g., aspartic, cysteine, serine, threonine, or metalloprotease inhibitors). Serine protease inhibitors are further grouped according to the presence of a particular structural motif [e.g., Bowman-Birk (Song et al. 2008), Kazal (Gebhard et al. 2004), or Kunitz (Conlon and Kim 2000)], but previously undescribed motifs also exist among anuran skin protease inhibitors (Chen and Shaw 2003).

The distribution of protease inhibitors in the skin secretion remains poorly characterised across anurans. From the first such compound to be identified – *Bombina* skin trypsin inhibitor (BSTI) from *Bombina bombina* (Mignogna et al. 1996) – subsequent studies revealed the presence of structurally conserved homologues in several congeneric species (Lu et al. 2008; Lai et al. 2002; Chen and Shaw 2003). More distantly, protease inhibitors have also been reported in the skin secretions of the neobatrachian frogs *Odorrana grahami* (Li et al. 2008; Han et al. 2008), *Odorrana versabilis* (Song et al. 2008) and *Lithobates areolatus* (Ali et al. 2002) (all Ranidae), the Giant Monkey Frog *Phyllomedusa sauvagii* (Hylidae) (Gebhard et al. 2004), as well as the Asian Toad *Bufo gargarizans* (Bufonidae) (Zhao et al. 2005a, b). All these latter species are also known to store different classes of bioactive peptides in their granular glands (see literature in the citations above), the exception being *B. gargarizans*, which, like other bufonids, instead produces a poison comprising steroid-like compounds and biogenic amines (Liu et al. 2010; Erspamer 1994).

Indeed, the co-production of protease inhibitors with other biologically active compounds in the skin secretion seems to be the rule among anurans. For instance, although AMPs are not present universally throughout anurans, they are present in virtually all species that possess protease inhibitors. The exception in this regard is the family of Narrowmouth Toads (Microhylidae), where the species that have been investigated apparently lack most if not all the aforementioned peptide classes in their skin secretions (see Table 1). To date, only serotonin and histamine have been identified in two microhylid species (Erspamer 1994; Roseghini et al. 1976) and, more recently, polypeptides with the ability to inhibit the protease trypsin in *Kaloula pulchra* and *Dyscophus guineti* (Conlon and Kim 2000; Zhang et al. 2010), with the last protein being classified as a member of the Kunitz/bovine pancreatic trypsin inhibitor family (BPTI).

The curious lack of bioactive peptides in the skin secretions of microhylid frogs, in contrast to the peptide-rich secretions of other frog species, begs the question as to the function of the protease inhibitors in these animals, if not across anurans in general. Three main hypotheses exist in this regard. The first promotes an indirect role of the inhibitors for chemical defence by preventing degradation of the endogenous host-defence peptides produced by the exocrine apparatus (Chen and Shaw 2003; Mignogna et al. 1996). Here, inhibition of proteases from either the frog itself or the invading pathogens is thought to be the main purpose of these peptides. Importantly, the former role presents a mechanism to control and mediate the processing of the biosynthetic precursors of host-defence peptides by balancing the activity of these endopeptidases and their respective inhibitors (Chen and Shaw 2003; Mignogna et al. 1996). A second hypothesis holds that the protease inhibitors possess antimicrobial activity (Conlon and Kim 2000) based on the observation that bovine aprotinin, a BPTI-related proteinase inhibitor with a Kunitz domain, exhibits inhibitory activity against a wide range of Gram-positive and Gram-negative microorganisms because of its cationic and hydrophobic properties (Pellegrini et al. 1992). Indeed, the Kazal inhibitor isolated from *P. sauvagii* showed slight antimicrobial activity against the Gram-negative *Escherichia coli* (Gebhard et al. 2004). Given that AMPs are apparently absent in microhylids including *D. guineti*, the protease inhibitors would thereby adopt a function analogous to them in this family (Conlon and Kim 2000). A third hypothesis holds that the adhesive secretions used by microhylids (and several other amphibian species) provide an efficient antipredator mechanism *per se* such that these animals tend to be devoid of any additional chemical weapons or, minimally, produce odorous, repelling agents (Evans and Brodie 1994). Important in this regard is the fact that the molecules responsible for the adhesive character of the skin secretion have not yet been identified.

With the present study, we aim to provide a better understanding of the chemical defence in Microhylidae, especially given that previous investigations might have been limited here by their focus on either biogenic amines (Roseghini et al. 1976) or peptides (Erspamer et al. 1986; Erspamer 1994). Using the Madagascan Tomato Frog *D. guineti* as an exemplar for the group, we re-investigated the defensive skin secretion of this frog using "shotgun" cloning of a skin secretion-derived cDNA library to present the first molecular data from the glandular transcriptome of any member of Microhylidae. We further interpret these data in the context of the distinct, adhesive skin secretion of this species as

well as their defensive behaviour of inflating their bodies (personal observation) to present a larger body size to an attacking predator (Duellman and Trueb 1994) to provide a comprehensive overview of the defensive tools of *D. guineti*.

Materials and methods

Animals and acquisition of the skin secretion

Two male specimens of *D. guineti* kept at Belfast Zoological Gardens and at the Thüringer Zoopark Erfurt were used for harvesting skin secretions, with the respective zoo management permitting the experiments in accordance with local and national guidelines. The first individual was electrically stimulated by mild transdermal electrical stimulation (5 Vdc, 4 ms pulse-width, 50 Hz) for three periods, each with a duration of 10 s (Tyler et al. 1992), whereas the second was stimulated by manual massage. The abundant, white, viscous skin secretion that resulted was subsequently washed off from the dorsal skin surface with Milli-Q water and collected in a sterile glass beaker. Samples were transported under chilled condition and snap-frozen with liquid nitrogen immediately after arrival at Queen's University. The lyophilisate was stored at -20 °C until analysis.

Molecular cloning of a cDNA library derived from the skin secretion of *Dyscophus guineti*

Five milligrams of lyophilised skin secretion from *D. guineti* were placed into 1 ml of cell lysis/mRNA stabilisation buffer, with polyadenylated mRNA being isolated from the buffer using magnetic oligo-dT beads as described by the manufacturer (Dynal Biotech, UK). Subsequently, the isolated mRNA was reverse-transcribed using a SMART-RACE kit (Clonetech, UK) to obtain a cDNA library that was subjected to 3′-RACE PCR using the supplied nested universal primer (NUP) and a degenerate sense primer (S1; 5′-CCIGCIGARGTIT-GYTTYYT-3′) designed according to the reported N-terminal amino-acid sequence (-PAEVCFL-) of the protease inhibitor from *D. guineti* (Conlon and Kim 2000).

The PCR was performed as follows: an initial denaturation step of 60 s at 94 °C; 35 cycles of denaturation of 30 s at 94 °C; primer annealing with a temperature gradient of 30 s at 54 °C, 56 °C, and 58 °C each; and finally extension of 180 s at 72 °C. The resulting PCR fragments of approximately 500 bp were purified (PCR purification Kit, Marligen Biosciences), cloned using a pGEM-T vector system (Promega Corp.), and sequenced using an ABI 3100 automated sequencer.

In a second step, two specific antisense primers (AS1: 5′-GAAGCTCAGCAGGATCACTGATT-3′; AS2: 5′-CAGGA GATCTTACAGGCGAGTA-3′) were designed according to an apparently conserved site within the 3′-untranslated region of the initial transcript obtained from 3′-RACE PCR. Thereafter, 5′-RACE reactions were performed using the NUP combined with either the AS1 or AS2 primers; subsequent purification, cloning, and sequencing were performed as described above.

All full-length cDNA sequences obtained were translated using Expasy (Swiss Institute of Bioinformatics), and the identified open-reading frames were then subjected to online

Table 1 Reported skin secretion compounds from microhylid frogs

Inclusive taxon	Species	Compounds identified from skin secretion	Reference
Asterophryinae (Southern Philippines, Sulawesi, and Bali, eastward through Indonesia and New Guinea to New Britain and extreme northern Australia; Moluccas)	*Albericus variegatus*	None	Roseghini et al. 1976
	Austrochaperina pluvialis	None	Roseghini et al. 1976
	Cophixalus cryptotympanum	5-HT (= serotonin)	Roseghini et al. 1976
	Cophixalus ornatus	None	Roseghini et al. 1976
	Hylophorbus rufescens	Histamine	Erspamer 1994
	Hypopachus variolosus	None	Erspamer et al. 1986
	Xenorhina rostrata	None	Roseghini et al. 1976
Dyscophinae (Madagascar)	*Dyscophus guineti*	Trypsin inhibitor	Conlon and Kim 2000
Microhylinae (Eastern Asia from India and Korea to the Greater Sunda Islands)	*Kaloula pulchra*	Trypsin inhibitor	Zhang et al. 2010
Gastrophryinae (North and South America)	*Gastrophryne carolinensis*	None	Erspamer et al. 1986

BLAST analysis (blastp) at the National Center for Biotechnology Information (NCBI) website for preliminary identification and to check for possible contamination.

Peptide purification and identification

Four milligrams of the lyophilised skin secretion from the male individual were dissolved in 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water (1 ml) before being cleared of microparticles and other debris by centrifugation (13,000 rpm; 10 min). The supernatant was directly injected into a reverse phased HPLC system fitted with an analytic column (Luna C18; 250×4.6 mm, Phenomenex, UK) using a gradient ranging from 0.05/99.95 (v/v) TFA/water to 0.05/29.95/70 (v/v/v) TFA/water/acetonitrile over 240 min at a flow rate of 1 ml/min. Absorbance was constantly monitored at λ214 nm and all fractions (1 ml) were collected for further analysis. Thereafter, 100-µl aliquots of each fraction were lyophilised and re-dissolved in phosphate-buffered saline (PBS) to assay their ability as protease inhibitors (see section below).

All fractions were further analysed using MALDI-TOF on a Voyager DE mass spectrometer (PerSeptive Biosystems, MA, USA) in positive detection mode using alpha-cyano-4-hydroxycinnamic acid as the matrix. The masses observed from fractions with inhibitory activity were compared with the mass calculated for the expected protease inhibitor (Conlon and Kim 2000).

Determination of inhibitory and antimicrobial activity

Inhibitory activity of the HPLC fractions was tested against two serine proteases, trypsin and the "trypsin-like" thrombin. For the former, a 0.1 µM solution of trypsin dissolved in 1 mM HCl was prepared from which 10 µl was transferred into each well of a 96-well microtitre plate containing 180 µl of a 50 mM substrate solution (Phe-Pro-Arg-AMC; obtained from Bachem, Switzerland) and 10 µl of the reconstituted chromatographic fraction from Peptide Purification and Identification in 10 mM PSB (containing 2.7 mM KCl and 137 mM NaCl, pH7.4). Each test of inhibitory activity was carried out in duplicate. The rate of substrate hydrolysis was monitored continuously at 37 °C by measuring the increase in fluorescence at 460 nm that resulted from the production of 7-amino-4-methylcoumarin (AMC; excitation 355 nm) in a CYTOFLUOR® multiwell plate reader series 4000 Fluorimeter (BMG Labtech, Germany).

Similarly, we tested for inhibitory activity against thrombin (Sigma-Aldrich, USA) using a substrate with a typical thrombin cleavage site (Boc-Val-Pro-Arg-AMC; Bachem, Switzerland). The substrate was initially dissolved in DMF to obtain a 10 mM stock solution that was further diluted with a 200 mM Tris-HCl buffer containing 100 mM NaCl and 1 mM $CaCl_2$ (pH7.8). Analogous to the trypsin assays, 180 µl of the 50 µM substrate solution was placed into each well of a 96-well microtitre plate together with 10 µl of the reconstituted HPLC fractions + PSB and a 10 nM thrombin solution. The rate of substrate hydrolysis was monitored as described above for trypsin.

Antimicrobial activity was tested using an agar diffusion test. A sterile filter plate (1 cm in diameter) was placed on a nutrient-bouillon medium (5 g pepton:3 g meat extract:15 g agar in 1 l distilled water; pH7) that was inoculated with either *Bacillus subtilis* (DSM 10) or *Escherichia coli* (K12, DSM 498). Subsequently, the filter plate was soaked with 10 µl of 5 mg lyophilized secretion reconstituted in PBS. Plates for both test microorganisms were incubated for 72 h at 30 °C. As a negative control, we used 10 µl PBS.

Results

The skin secretion of *Dyscophus guineti*

In contrast to the typically soft and powdery samples obtained from most other frog species, the freeze-dried skin secretion of the Tomato Frog was very compact and hard. During preparation of the sample for the HPLC analyses (see Peptide Purification and Identification), we found that the lyophilisate was only partial soluble in 0.05 % TFA. We therefore applied trypsin to the remaining gel-like clot from the centrifugation step to assess whether it contained proteins that ought to be hydrolysed by the proteolytic activity of this enzyme. Although the clot was not entirely digested, we observed considerable digestion of the clot after 24 h of incubation with 1 mM trypsin (Sigma-Aldrich, USA) (data not shown).

"Shotgun" cloning of the skin secretion transcriptome

From the cDNA library, we obtained the complete biosynthetic precursor of the putative trypsin inhibitor. It possessed an open-reading frame of 78 amino-acid residues comprising an N-terminal putative signal peptide of 21 amino acids followed directly by a biologically active polypeptide that was unequivocally identified as the Kunitz trypsin inhibitor described previously (Conlon and Kim 2000) (Fig. 1). A blastp search of this sequence against GenBank revealed two hits to other anuran protease inhibitors: a chymotrypsin inhibitor from *Kassina senegalensis* (GenBank accession no. CBY65969; identity=33/81; E-value=3.0×10^{-12}) and a serine protease from *Rana chensinensis* (ACV66786; 52/82;

```
                                                  M  K  T  L  L  L  L  A
  1  GGGCAGCCTC CAACCTCTCC ATCAAACATG AAGACCCTCC TGCTTCTCGC
     CCCGTCGGAG GTTGGAGAGG TAGTTTGTAC TTCTGGGAGG ACGAAGAGCG
        V  I  V  F  S  S  F  W  S  F  S  S  S  S  P  A
 51  TGTTATTGTC TTCAGTTCCT TCTGGTCATT CAGCTCTTCA TCTCCAGCCG
     ACAATAACAG AAGTCAAGGA AGACCAGTAA GTCGAGAAGT AGAGGTCGGC
        E  V  C  F  L  P  K  E  S  G  L  C  R  A  R  A  L
101  AAGTTTGTTT CTTACCGAAA GAGAGCGGCC TCTGCAGAGC GCGCGCCCTG
     TTCAAACAAA GAATGGCTTT CTCTCGCCGG AGACGTCTCG CGCGCGGGAC
        R  Y  Y  Y  D  R  G  D  G  K  C  E  E  F  I  Y  G
151  CGCTATTACT ACGATAGAGG AGACGGAAAG TGCGAGGAGT TTATTTATGG
     GCGATAATGA TGCTATCTCC TCTGCCTTTC ACGCTCCTCA AATAAATACC
        G  C  G  G  N  G  N  N  Y  K  S  L  L  T  C  K
201  CGGCTGTGGA GGGAATGGAA ATAACTACAA ATCGTTACTC ACCTGTAAGA
     GCCGACACCT CCCTTACCTT TATTGATGTT TAGCAATGAG TGGACATTCT
        I  S  C  E  *
251  TCTCCTGCGA ATAATCAGTG ATCCTGCTGA GCTTCAGGAG CTAAGGCAAA
     AGAGGACGCT TATTAGTCAC TAGGACGACT CGAAGTCCTC GATTCCGTTT
301  ACTCCCCTGA AAATGCTCTG CTTCTGGGAG TGACAGCGGA CCTACACCCT
     TGAGGGGACT TTTACGAGAC GAAGACCCTC ACTGTCGCCT GGATGTGGGA
351  GACATAGAAG CTGGTCCACT TTAAACTGGG ATACGAAATT CCGGATAAAC
     CTGTATCTTC GACCAGGTGA AATTTGACCC TATGCTTTAA GGCCTATTTG
401  GCTGTTTGGG GCTCTGCTCT ATTCTGCATT TTTAATATAC TTTTCTGCAT
     CGACAAACCC CGAGACGAGA TAAGACGTAA AAATTATATG AAAAGACGTA
451  TAAACCTGAA CCAAAAAAAA AAAAAAAAAA AAAAAAAA
     ATTTGGACTT GGTTTTTTTT TTTTTTTTTT TTTTTTTT
```

Fig. 1 Nucleotide sequence of the precursor cDNA for the trypsin inhibitor isolated from *Dyscophus guineti*. The putative signal peptide (*double underlined*), mature peptide (*single underlined*), and stop codon (*asterisk*) are indicated

E-value=4.0×10^{-12}). Indeed, of the top hits (the top 25 of which are presented in Table 2), the majority were to different serine protease inhibitors, most frequently from the BPTI/Kunitz protease inhibitor family, albeit originating from a wide range of animals other than frogs (e.g., anthozoan cnidarians, spiders, insects, and several venomous snake species from the family Viperidae). In addition, the search also yielded a thrombin inhibitor from a tick and numerous tissue factor pathway inhibitors from several mammals and teleost fishes, for which the highest scores were often recorded. The high similarity of the isolated inhibitor from *D. guineti* to the latter two inhibitor families raises the possibility that this protease might also possess an antithrombin-like activity, which we tested in a parallel bioassay analogous to that which we used to establish trypsin inhibition (see section below).

The sequence of the identified protease inhibitor has been submitted to GenBank (accession no. JX294469).

Isolation and activity of the protease inhibitor

Apart from the putative Kunitz trypsin inhibitor, MALDI-TOF analyses did not detect any other peptides (e.g., antimicrobial peptides) in the skin secretion of *D. guineti* and the constituent HPLC fractions. Inhibition of trypsin activity was observed in fractions 86-89 from the reverse-phase HPLC (Fig. 2). Subsequent analysis with MALDI-TOF indicated a polypeptide in these fractions with an m/z of 6,331 Da (Fig. 3). Under the assumption that salt derivatives may have formed during preparation for analysis and that the polypeptide is indeed present as an Na^+ salt (24 Da), the corrected value agrees with the calculated masses of the translated open-reading frame obtained from molecular cloning (6,307 Da or 6,301 Da with disulfide bonds; see section above) and the previously reported sequence of the trypsin inhibitor obtained by Edman degradation (Conlon and Kim 2000).

The weak intensity of the peaks in both the HPLC chromatogram and the MALDI-TOF spectrum suggests that relatively low amounts of the active polypeptide are present in the skin secretion of *D. guineti*. Nevertheless, the proteolytic activity against trypsin in the respective fractions was high, although we reconstituted only 10 % of the fraction (Fig. 2b). By contrast, no proteolytic activity against thrombin was detected in any fraction, and, contrary to a previous report (Conlon and Kim 2000), no antimicrobial activity for the trypsin inhibitor was observed.

Discussion

Through "shotgun" cloning of the skin secretion transcriptome of the Tomato Frog, *D. guineti*, we were able both to confirm the presence of an apparent trypsin inhibitor from the Kunitz family of serine protease inhibitors and provide the first full-length cDNA sequence for it. A comparative analysis of this gene with other protease inhibitors known in Anura provides another example of the high degree of convergence present in the defensive system of these animals (König and Bininda-Emonds 2011). For instance, the cDNA encoding the trypsin inhibitor of *D. guineti* (as well as that from a hyperolid frog, *K. sengalensis*) shows little sequence similarity with those from the genus *Bombina*, although both share a similar structural architecture of a putative signal sequence followed directly by a single copy of the mature protease inhibitor (Chen and Shaw 2003; Lai et al. 2002; Lu et al. 2008; Mignogna et al. 1996). High sequence similarity is instead found with a transcript from the skin secretion of *Rana chensinensis*, with the entire gene differing slightly architecturally in that it exhibits three copies of the inhibitor. By contrast, the precursors of other ranid protease inhibitors are present as single copies, but structurally resemble AMP precursors through the intervening spacer peptide that is present between the N-terminal signal sequence and the C-terminal peptide domain (Li et al. 2008; Song et al. 2008). The similarity also extends to the sequence level in that the entire putative signal peptide encoded in these ranid protease inhibitor cDNAs is virtually identical to that from the neobatrachian AMP precursor motif (König and Bininda-Emonds 2011; Vanhoye et al. 2003; Song et al. 2008).

On a more functional level, our analyses also further confirm that the *D. guineti* trypsin inhibitor does indeed appear to be the only bioactive compound in the defensive secretion of this peculiar frog. Moreover, although a potent inhibitor of trypsin, this polypeptide showed no activity against thrombin (despite its sequence similarity with known antithrombin proteins) and, contrary to the hypothesis of Conlon and Kim (2000) based on theoretical but not experimental grounds (see Introduction), also did not display any antimicrobial activity.

In the case of the Tomato frog, these data are sufficient to reject the first two hypotheses mentioned in the Introduction explaining the possible function of this proteolytic polypeptide in the skin secretion of this species (e.g., protection of endogenous host-defence peptides versus a functional analogue of the missing AMPs) and provide impetus for alternative explanations. Although most of the small number of frog species known to express protease inhibitors in their skin secretions (Ali et al. 2002; Chen and Shaw 2003; Gebhard et al. 2004; Lai et al. 2002; Li et al. 2008; Lu et al. 2008; Mignogna et al. 1996; Song et al. 2008) also co-produce a wide range of host-defence peptides (Ali et al. 2002; Simmaco et al. 2009; Calderon et al. 2011; Conlon et al. 2006; Chen et al. 2006), microhylid frogs are the exception in that the latter class of compounds has never been documented in this family despite both *D. guineti* and *K. pulchra* being known to have a protease inhibitor (Conlon and Kim 2000; Zhang et al. 2010). Similarly, the lack of any antimicrobial activity of the trypsin inhibitor in *D. guineti* (as well as that for the trypsin inhibitor from *K. pulchra*; see Zhang et al. 2010) generally rules out a role for it as a substitute for the missing AMPs. That being said, a general anti-infective function of the trypsin inhibitor cannot be excluded entirely because it could act against extrinsic proteases produced by microorganisms to invade to host organism on this route (Chen and Shaw 2003). In addition, it remains that the antimicrobial ability of protease inhibitors among anurans has scarcely been tested explicitly to date.

It therefore remains a mystery why *D. guineti* (and *K. pulchra*) synthesises a large and reasonably costly 6-kDa endopeptidase inhibitor in the absence of any apparent host-defensive effect mediated by endogenous peptides, which in turn should be protected from degradation through the inhibitor. This is especially the case given that the primary defence of *D. guineti* would appear to be mechanical through its abundant, extremely viscous and sticky skin secretion (see Evans and Brodie 1994), which might be related to the extremely large, but unidentified polypeptides and proteins we indirectly observed in it. Moreover, the trypsin inhibitor also occurs at much lower concentrations than do most other anuran skin peptide classes with an explicit defensive function (e.g., AMPs or neuropeptides).

Table 2 Alignment of the protease inhibitor from *Dyscophus guineti* and the first 25 BLAST hits (blastp) from GenBank

Sequence	Accession no.	Source taxon
SPAEVCFLPKESGLCRARALRYYYDRGDGKCEEFIYGGCGGNGNNYKSLLTCKISCE	AFR78284	*Dyscophus guineti*
QAGSI.LE..VV.P.T.YFR.F.F.SET...TV.....E.....FET.RA.RAI.R	B2G331	*Heteractis crispa*
.SED..S..SDT.M...SKPMFF..HEK.E.AK.....Q....RF.TIEE.EST.G	ADV40356	*Latrodectus hesperus*
K.SSI.Q...VV.P...SLK..R..STT.Q....T....K..E..FITREV.QEN.I	EFN87118	*Harpegnathos saltator*
--S--.EQE.VV.P...AFR..FFNKAT.Q..L.....R..D..FGTKEE.ESV.L	ADV40281	*Latrodectus hesperus*
GRPAK.K..PDD.P....IPS..F..KTKT.K..M....E..E..FENIT..QEE.R	CAB55816	*Ixodes ricinus*
-.-.F.HF.RDP.P.KTYM...FHNTKSN....L.....H..K..F.TFDS.HYT.V	XP_003226823	*Anolis carolinensis*
.SG.R.YMKPKT.P.K.KYE.FH...K.LT.KP.........E..F.TKEE.QKA.K	AAY66790	*Ixodes ricinus*
CLNSI.S...NA.P.K.YFP....NSKT....K......K..Q..FQ.SKD.EQN.H	AFJ24834	*Schmidtea mediterranea*
-.---.LD.LDK.R.S.SIP....NTATKM......S....SS..FV.RQS.MDV.V	ADM64310	*Sciaenops ocellatus*
-----....LSK.F...HWV.....PSSRS.KS.V....D.....FH..NE.RLA.L	EFX87486	*Daphnia pulex*
-.---.LDLLDK.K.S.SIP....NTASKM......S....SS..FV.RRS.MDV.V	ACQ58107	*Anoplopoma fimbria*
-.---.YS..DE...S.NVT...FNPRHKA..A.N.T.....D..FVN.KD..RT.V	NP_877589	*Bos taurus*
-.---.YS..DE...S.NVT...FNPRHKI..A.T.T.....D..FV.MQD..QV.T	XP_003407191	*Loxodonta africana*
DFE--.KAAP.T...K.SFE.WFFNAAS.E.....D...ENKEE.EFA.K	BAH02683	*Haemaphysalis longicornis*
-.---.LD.LDK.K.S.SIP....NAATKR....A.S....SS..FV.RQS..DV.V	XP_003969359	*Takifugu rubripes*
DVPKF.D.SPDP.P.FGHMNH....PSTNS.KP.V....Q.....FQTVKE.E.A.R	CBY65969	*Kassina senegalensis*
-.---.YS..DE...S.NVT...FNPRHKA..A.T.T.....D..FVNRKD..QV.A	XP_001492819	*Equus caballus*
.GNSA.T...KV.D...AMP.VFFNSQS....D.........A..FHTMEE..KA.M	XP_001621919	*Nematostella vectensis*
DHPVF.Y..ADP.I.K.HKP.F..NPASN..K..F.......A..F.TRDE.HHT.V	P0DKL8	*Vipera renardi*
-.---.LD.LDR.G.A.SIP....NSASRM..Q...S....SS..FI.KQS.MDV.A	ACI69296	*Salmo salar*
-.---.LD.LDK.G.A.SIP....NSASRMR.Q...S....SS..FI.RQS.MDVRA	ACO08416	*Oncorhynchus mykiss*
--.--.D..SD..P.EVYIP.F....ETKT.KD......Q.....FLTKED.ERT.K	ACV66786	*Rana chensinensis*
-.---.LD.LDK.G.A.SIP....NSASRM..Q...S....SS..FI.RQS.MDV.A	NP_001158586	*Oncorhynchus mykiss*
-.---.YS..DE...S.NVT...FNPRHKA..A.T.T.....D..FVN.KD..RT.V	XP_004007771	*Ovis aries*
--A--.QQEK.V...K.YMP.FFFNKHT.............S..FN..ED.EAV.Y	ADV40132	*Latrodectus hesperus*

Gaps (–) have been introduced to increase overall similarity. Dots designate amino acid residues that are identical to the sequence of *D. guineti* trypsin inhibitor (in bold).

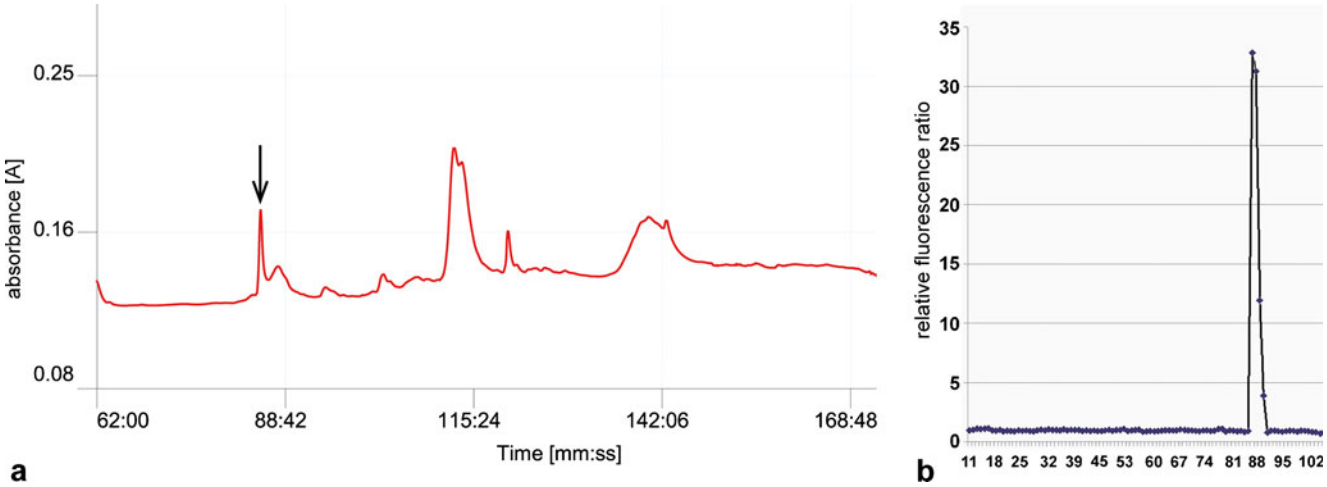

Fig. 2 Reverse phase HPLC chromatogram of the skin secretion from *Dyscophus guineti* (**a**). For the fraction in (**a**) marked with an *arrow*, a clear inhibition of trypsin activity was observed (**b**)

We suspect that the trypsin inhibitor, despite its observed lack of activity against thrombin, must be a more general serine protease inhibitor ("serpin") if it indeed fulfils a direct defensive function. Given that trypsin is produced in the digestive tract of vertebrates, a pure trypsin inhibitor would merely delay digestion of the frog, but not it being swallowed in the first place. Instead, like other serpins, the trypsin inhibitor could also be effective against a broader range of chymotrypsin-like proteases as well as some cysteine proteases. Noteworthy is that serpins are involved in many regulative, physiological processes, including the processing and release of peptide hormones, blood coagulation, and complement fixation. In addition, they are involved in the pathogenesis of numerous diseases, which is why they also have

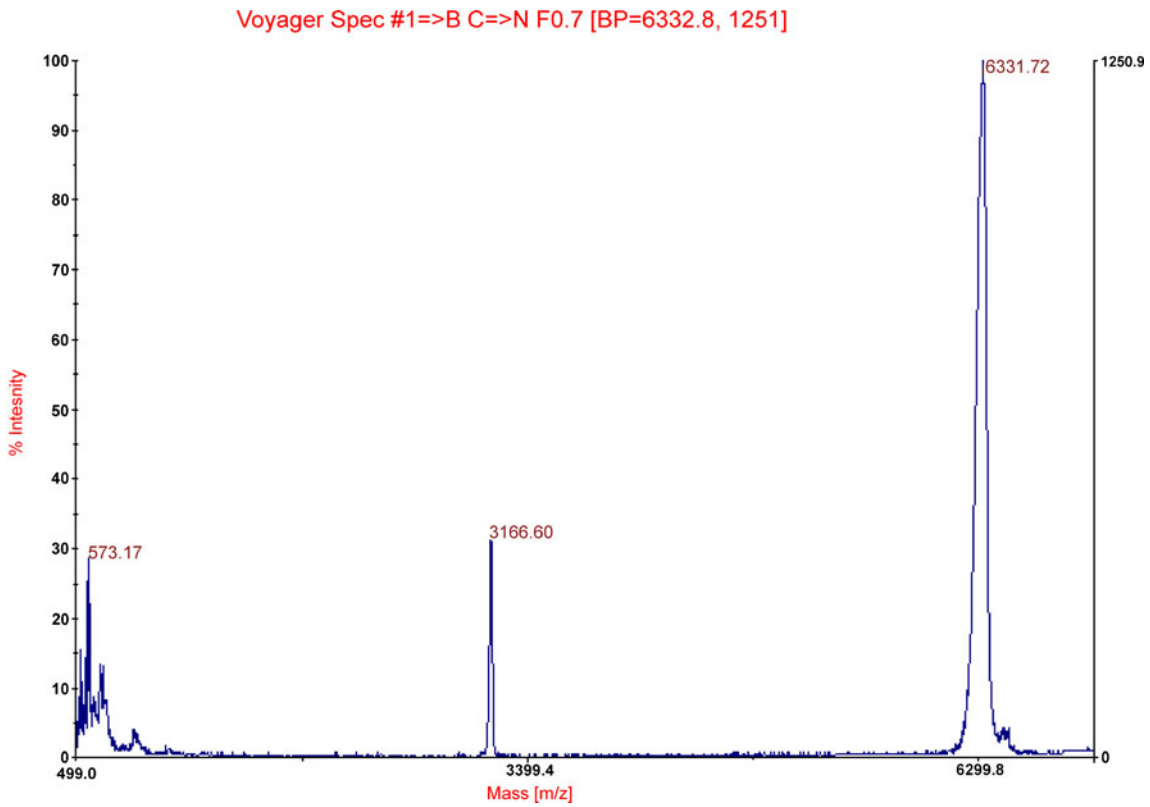

Fig. 3 MALDI-TOF mass spectrum of the protease inhibitor-containing fraction

become of great interest for pharmacological research (Chen and Shaw 2003). Even so, and in part because of this diversity of potential functions, it remains unclear what the target of the trypsin inhibitor in *D. guineti* is.

It is possible, however, that the trypsin inhibitor plays an important enzymatic role in the mechanical defence provided by the skin secretion by preventing polymerization of the large proteins in the secretion during the storage period in the cutaneous glands. Given the compact nature of the glands, a high concentration of the inhibitor would not be required to hold back the enzymatic activity of its substrate. However, once the secretion is discharged, for example in response to a predatory attack, the trypsin inhibitor becomes overwhelmed because of its low concentration, either by the salivary proteases of the ingesting predator or simply through the spatial dispersal of the secretion *per se*. This, in turn, results in fast agglutination of the secretion and its characteristic, adhesive character. An analogous process underlies the formation of barnacle cement, which derives from a secreted adhesive that is hypothesized to underlie polymerization initiated by trypsin-like proteolytic activation that is biochemically similar to blood clotting and the activation of zymogens (Dickinson et al. 2009).

More direct although still circumstantial evidence for this general scenario comes from an analogous defensive reaction reported from the salamander *Batrachoseps attenuatus*, which is sufficient to temporarily immobilize its serpent predator (Duellman and Trueb 1994; Arnold 1982). Similarly, the survey of Evans and Brodie (1994) provides evidence for a number of amphibian species that produce glue-like secretions to adhere the predator to the substrate, thereby enabling the frog to escape. The latter study included *D. guineti* where the adhesive nature of the skin secretion – together with that of the closely related *Dyscophus antongilii* – was shown to be the strongest of those studied as quantified by measuring the tensile strength of the secretion using a force transducer (Evans and Brodie 1994). Importantly, possible predators of *D. guineti* include snakes from the genus *Madagascarophis* (Lamprophiidae) (F. Andreone, pers. comm.), which are close relatives of the venomous Elapidae (Pyron et al. 2011). Snake venoms are well known to contain both numerous serine proteases (or other cleaving enzymes) to prevent defensive reactions against the venom in the blood vessels of the prey to assure toxic reactions as well as protease inhibitors to prevent premature activation of the venom (Lu et al. 2005). However, given that the skin secretion of *D. guineti* can already take on its characteristic adhesive nature in the absence of a true predator, it could be that the trypsin inhibitor functions primarily to prevent polymerization of the defensive polypeptides during their storage. However, it cannot be ruled out that the inhibitor may also act to inhibit the salivary proteases of a predator that would digest the as yet unidentified organic constituents responsible for the adhesive character of the skin secretion. As we show here, the latter can be trypsinised after isolation of the protease inhibitor. Clearly, any delay in digesting the glue-like components will prolong this defensive mechanism, which may include adhering the predator to the substrate (e.g., leaves) or its own body, thereby distracting it to permit the frog to escape as demonstrated by Evans and Brodie (1994). A potentially key piece of evidence in support of our proposed hypothesis for the function of the protease inhibitor would be to identify the as yet unknown organic molecules responsible for the adhesive nature of the skin secretions in *D. guineti* and how they are acted upon by the trypsin inhibitor.

Conclusions

Although the exact function of the trypsin inhibitor from *D. guineti* remains unknown, we hypothesize that this enzyme potentially functions as a more general serine protease inhibitor despite its lack of activity against thrombin. Characteristic for this species is the highly viscous skin secretion, which contains a strong, glue-like adhesive for which the responsible compounds remain unidentified. It seems plausible that the trypsin inhibitor acts indirectly as a regulator to maintain a soluble state of the secretion during storage in the cutaneous glands to facilitate its fast discharge whereupon a rapid biochemical reaction occurs to yield the characteristic skin secretion. Thus, the major defensive role of the Tomato Frog's skin secretions appears to be mechanical through adhesion rather than chemical through antimicrobial activity or toxicity. Thus, ingestion by potential predators, such as snakes, is impeded by gluing the mouth closed through the combination of massive bulk discharge of the viscous secretion and body inflation as observed in this and other microhylid species.

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