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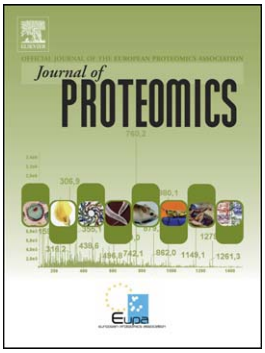

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The chains of the heterodimeric amphibian skin antimicrobial peptide, distinctin, are encoded by separate messenger RNAs

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Abstract

Using a primer to a conserved nucleotide sequence of previously-cloned skin peptides of *Phyllomedusa* species, two distinct cDNAs were "shotgun" cloned from a skin secretion-derived cDNA library of the frog, *Phyllomedusa burmeisteri*. The two ORFs separately encode chains A and B of an analog of the previously-reported heterodimeric peptide, distinctin. LC-MS/MS analysis of native versus dithiotreitol reduced crude venom, confirmed the predicted primary sequences as well as the cystine link between the two monomers. Distinctin predominantly exists in the venom as a heterodimer (A-B), neither of the constituent peptides were detected as monomer, whereas of the two possible homodimers (A-A or B-B), only B-B was detected in comparatively low quantity. *In vitro* dimerization of synthetic replicates of the monomers demonstrated that besides heterodimer, both homodimers are also formed in considerable amounts. Distinctin is the first example of an amphibian skin dimeric peptide that is formed by covalent linkage of two chains that are the products of different mRNAs. How this phenomenon occurs *in vivo*, to exclude significant homodimer formation, is unclear at present but a "favored steric state" type of interaction between chains is most likely.

Keywords: Amphibian skin peptides; cDNA cloning; distinctin; intramolecular disulfide bonds; peptidomics.

1. Introduction

Amphibians are known to release bioactive compounds from their skin granular glands that include alkaloids, steroids, biogenic amines, proteins and peptides [1, 2]. Many hundreds of different peptides, differing in size, charge, hydrophobicity, conformation, primary structure, as well as in post-translational modifications, have been demonstrated in frog defensive skin secretions.

As already reported by Vittorio Erspamer, one of the original frog skin peptide researchers, frogs from the genus *Phyllomedusa* have skins that are "a treasure trove of biologically-active peptides" [3], due to the large quantities and structural diversity of the peptides that he found

in members of this taxon. Several *Phyllomedusa* skin peptides bear striking identities to mammalian (neuro)peptides [3, 4], and exhibit bioactivities in mammalian systems that are consistent with interaction with and activation of endogenous receptors. Other peptide classes include highly-potent antivirals and antimicrobials that are effective against bacteria as well as protozoa (recently reviewed [5, 6]). These rich sources of bioactive peptides are of particular interest to pharmacologists who are continuously searching for potential novel drug leads from unusual sources. Peptides thus far isolated from phyllomedusine skin, belong to diverse families, among which the dermaseptins and phylloseptins represent the largest both in terms of the numbers of peptide sequences identified and in the numbers of species in which they have been found [6]. The emergence of high throughput techniques such as tandem mass spectrometry based "peptidomics" and cDNA cloning have substantially assisted in enlarging the collection of sequences of bioactive (frog skin) peptides. Performance of high resolution mass spectrometry linked to efficient and robust (nano)HPLC or UPLC, is a very powerful combination strategy to collect (partial) amino acid sequence information straight from complex peptide mixtures. The data thus generated can then be validated and/or completed as soon as the nucleic acid sequences of the corresponding mRNAs become available. The latter has been facilitated by use of an efficient molecular cloning approach, developed by Chen and coworkers [7], based on the use of magnetic oligo(dT) beads to extract polyadenylated mRNA sequences directly from only a few milligrams of the actual frog skin secretion. This technique does not require dissected tissue(s) for mRNA extraction, and hence does not necessitate sacrificing secreting donor specimens. Indeed, harmless 'gland milking' appears sufficient to collect frog skin secretions rich in both peptides and (their) corresponding messenger RNAs.

One successful strategy to "shotgun" clone novel skin peptide precursor-encoding cDNAs from frog skin cDNA libraries, has proven to be possible by the design of primers from highly-conserved nucleic acid sequences derived from skin peptide precursor-encoding cDNAs from related frog species [8, 9].

We here report the cloning and full structural characterization, directly from lyophilized *P. burmeisteri* skin secretion, of two peptide precursor-encoding cDNAs and their encoded peptides which are homologous to chains A and B from the heterodimeric peptide, distinctin, originally isolated from the skin of the related specie, *P. distincta* [10]. Differential analysis of native and reductively-alkylated *P. burmeisteri* distictin demonstrated that the intermolecular disulfide bridge between both chains is an endogenous and specific post-translational modification (PTM).

Among the PTMs that are characteristic for amphibian skin peptides, disulfide bonds are not uncommon features. However, most of the cystines in frog peptides are intramolecular. The so-called *Rana* box, for example, is a conservative C-terminal motif of 5-8 residues between a cystine [11]. This is typical for certain classes of antimicrobial peptides that are active against Gram-positive/-negative bacteria and the yeast, *Candida albicans*, and is found in peptide groups including the brevinins, gaegurins, nigrocins, odoranains, esculentins, ranalexins, dybowskins, japonicins, palustrins and ranatuerins [12-16]. Other conserved internal disulfide-bridged domains in frog skin peptides are known, such as in the skin calcitonin-gene related peptide and the Kazal protease inhibitor peptides, found in *P. bicolor* and *P. sauvagei,* respectively [17, 18].

Intermolecular disulfide bonds, however, are quite rare in amphibian skin peptides. The only example so far is distinctin, a 5.4 kDa heterodimer composed of two different peptide chains containing 22 and 25 residues, respectively, that was originally identified in the skin secretion of the phyllomedusine frog, *P. distincta*. It has antimicrobial activity against Gram-negative and Gram-positive bacteria [10, 19, 20], and the dimerization was found to enhance the peptides' bioactivity with respect to that of each monomer. In this context, heterodimeric peptides may represent a new class of amphibian skin peptide with potent biological/pharmacological activity that relies upon the formation of intermolecular complexes. This has inspired other researchers to actually engineer disulfide bridges into natural monomeric and linear amphibian skin peptides to form such complexes. Bioassays indeed subsequently confirmed that synthetic heterodimers, such as between magainin 2 and PGLa, and between magainin and its analogue pexiganan (i.e., MSI-78), showed a greatly enhanced antimicrobial activity when compared to the original native (monomeric) peptides [21].

2. Materials and Methods

2.1. Skin secretion collection

About 10 specimens of *Phyllomedusa burmeisteri*, one of the Brazilian walking leaf frogs, were captured during expeditions (January 2009) in the Pacotuba Forest (Cachoeiro de Itapemirim City), and at a local farm (Brunoro's, at Venda Nova do Imigrante City), both in the State of Espírito Santo (Brazil). Skin secretion samples were collected in the field by gentle transdermal electrical stimulation, essentially as described by Tyler and coworkers

[22]. The secretions were jet-washed from the sampled frog with deionized water, after which the donors were immediately released back into their natural environment. Skin secretions from several individuals (males and females) were filtered over cellulose acetate (0.2 µm pore size), pooled, frozen, lyophilized, and stored at -20 C prior to analysis.

The required permit to access the genetic heritage information was obtained from the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA license number 010453/2010-5).

2.2. Peptide separation and mass spectrometry

One mg of lyophilized crude skin secretion was diluted in 25 mM NH_4HCO_3. This sample was divided in two. One part was reduced (2 mM dithiothreitol (DTT)) and alkylated (4 mM iodoacetamide (IAM)). Equivalents of 200 micrograms of both samples – native and reduced – were separately analyzed by HPLC (Waters 2695 Alliance, Manchester, UK), on a C4 column (Reprosil C4, 5 µm particles, 2 mm x 150 mm, Dr. Maish, Germany) coupled on-line to a tandem mass spectrometer. The column was eluted (solvent A, 0.05% TFA, 0.1 M HAc; and B, 0.05% TFA, 0.1 M HAc in acetonitrile) by a linear gradient from 0 to 60% B at 0.75 % min^{-1}, and the eluate was directly nanosprayed into a Q-TOF (QTof Premier, Waters, Manchester, UK). Of the crude secretion, 0.1 µg was also analyzed by nanoLC using a C8 capillary column (Reprosil-Pur 120 C8, 5 µm particles, 150 mm, Dr. Maish, Germany) coupled to a linear trap-Orbitrap (Orbitrap Velos, ThermoFisher Scientific, Bremen, Germany).

To facilitate the location of peptides containing cystine, PTM-driven differential peptide displays were generated by MSight (SIB, Geneva, Switzerland) as described earlier [23]. This proved an elegant way to assess the presence of the various distinctin dimers and monomers in both untreated and reduced secretions.

For extensive targeted collision-induced fragmentation analysis of all distinctin ions, the respective peaks were manually selected at their retention times in a separate replicate LC MS/MS run.

2.3. Molecular cloning of distinctin peptides chains A and B

Five mg of lyophilized skin secretion were dissolved in 1 ml of cell lysis/mRNA stabilization solution (Dynal, UK). Polyadenylated mRNA was isolated using magnetic oligo-dT beads as

described by the manufacturer (Dynal Biotech, UK). The isolated mRNA was subjected to 5' and 3'- RACE procedures to obtain full-length peptide precursor nucleic acid sequence data using a SMART-RACE kit (Clontech UK). Briefly, the 3'-RACE reactions employed a nested universal primer (NUP - supplied with the kit) and a degenerate sense primer (N2-S1; 5'-ACTTTCYGAWTTRYAAGMCCAAABATG-3'), that was complementary to a conserved sequence 5' to the putative signal peptide (including the startcodon ATG). This primer had been successfully used to clone different skin secretion peptides from other *Phyllomedusa* species [8, 9]. 3'-RACE reaction products were gel-purified, cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an automated DNA sequencer (ABI 3100).

2.4. Peptide synthesis and oxidation reactions

A and B chains were synthesized by standard solid-phase Fmoc chemistry on an automated peptide synthesizer (Protein Technologies PS3, Tucson, AZ, USA).

Molar equivalents of synthetic chains A and B were 'incubated' (one week at RT) with themselves or in combination in two different conditions: i) in H_2O and ii) in 50 mM NH_4HCO_3 pH 8.0, containing 5% DMSO. In the H_2O condition the synthetic chains were merely diluted in water individually and A plus B mixed, and analyzed by LC MS/MS. In the other condition the three possible combinations of chains (only A, only B and A plus B) were induced to oxidize in bicarbonate buffer in 5% DMSO pH 8.0, and measured by MS.

3. Results

3.1. "Shotgun" cloning of distinctin chain precursor-encoding cDNAs

To amplify the distinctin precursor-encoding mRNA sequences present in a cDNA library constructed from lyophilized *P. burmeisteri* skin secretion, a degenerate primer pool designed to a conserved nucleotide sequence upstream of the putative signal sequence of previously cloned *Phyllomedusa* skin peptides, was employed for its interrogation [8, 9]. Two full size preprodistinctin cDNAs were thus "shotgun" cloned yielding two different open reading frames (ORFs) with separate start and stop codons.

The prepropeptide sequences can be predicted on the basis of putative prepropeptide convertase motifs and comparisons with similar peptides reported in the literature [7]. The open-reading frame (ORF) of the cDNA encoding the precursor of chain A, contains 69

amino acid residues, the first 22 of which, comprise a putative signal peptide, followed by a 25-residue acidic 'spacer' peptide and finally the 22-residue mature distinctin A chain peptide. The ORF of the cDNA encoding the precursor of chain B contains 67 residues, that includes a 20-residue putative signal peptide, a 22- residue acidic spacer and a 25-residue mature distinctin B chain peptide (Fig. 1).

The mature distinctin A- and B-chain peptide sequences are structurally very similar to those described for the original *P. distincta* peptide [10]. A significant difference in the primary structure is at residue 7 of the A-chain, which is an alanine (A) in the *P. burmeisteri* peptide, whereas the *P. distincta* homolog has a proline (P) in this position.

3.2. Identification of homo- and heterodimers by MS

MS analysis of crude *P. burmeisteri* skin secretion confirmed the existence of distinctin predominantly as a heterodimer (Fig. 2). The amino acid sequences of the chains were determined by collision-induced dissociation (CID) of both monomers that had been generated by reduction and alkylation of the venom components. MS/MS analysis was carried out using Q-TOF MS as well as by linear ion trap-Orbitrap tandem mass spectrometry. *De novo* sequencing of both chains, fully confirmed the sequences predicted from the "shotgun" cloned precursor cDNAs: a 22-residue mature A-chain: ENREVPAGFTALIKTLRKCKII, and a 25-residue mature B-chain, NLVSGLIEARKYLEQLHRKLKNCKV. In the DTT-treated skin secretion, as expected, no dimers remained (neither homo- nor heterodimers), which can easily be seen in the PTM-driven differential peptide display of reduced versus untreated (native) sample (Fig. 3).

The distinctin heterodimer, (A-B, *m/z* 779.45 (7+ charge state)), in the zoomed-in area of Fig. 3) was present exclusively in the native sample (complete absence in the DTT-treated venom). On the other hand, the monomeric A-chain (*m/z* 625.96) and B-chain (*m/z* 738.92, four times charged), were entirely absent from crude native skin secretion indicating that in this, they are cross-linked (by cystine bonds) and that the native heterodimer was efficiently reduced by the DTT treatment.

Likewise, targeted analysis identified the homodimer B-B in the native skin secretion (high resolution FTMS analyses; Fig. 2A). The ion intensity of the heterodimer is clearly higher than homodimer B-B, suggesting heterodimeric distinctin is present in a higher concentration than the homodimer B-B. No monomeric chain A or B, or any of the homodimer A-A, were detected in native skin secretion.

The heterodimer was observed at *m/z* 909.19 $[M + 6H]^{6+}$, or parent mass 5,449.12 Da. This exactly equals the sum of the masses of chains A and B, minus the mass of 2 protons (disulfide formation from two cysteines). CID of this heterodimer ion precursor indeed yielded both b- and y-ions (as well as immonium ions) representing the N-terminal sequences of chains A and B (peaks highlighted in Fig. 2B). Sequence ions representing residues close to the cystine connection could not be identified, due to the covalent bond between the chains. Similarly the homodimer B-B, with an observed molecular mass of 5,901.34 Da was selected and CID fragmented, showing only chain B amino terminal sequence ions. The precursor mass as well as the occurrence of charge states higher than 8 (protonatable residues (R, K, H, and the amino terminus)) proved that it is indeed the covalent dimer.

3.3. In vitro *oxidation of synthetic replicates of distinctin A- and B – chains*

In an attempt to answer the intriguing questions as to how/why two peptide chains originating from two different ORFs preferentially form heterodimers, synthetic replicates of the peptide monomers were made and their *in vitro* dimerizations were studied by high resolution nano LC MS/MS.

Synthetic A- and B-chains were either left to react in H_2O or in bicarbonate buffer containing 5% DMSO. In H_2O, no dimer was formed (Fig. 4A-C). However, air oxidation of the chains in bicarbonate buffer containing 5% DMSO, induced partial dimerization. Chain A appeared nearly 100 % oxidized when left to react with itself (Fig. 4D), whereas the oxidation to form homodimer B-B, was incomplete (Fig. 4E). The oxidation reaction containing both chains, resulted in the formation of all possible dimers: heterodimer A-B, homodimer B-B and a little homodimer A-A. Only traces of chain A and B monomer remained (Fig. 4F). These oxidation reactions showed no clear preferential dimer formation. They also demonstrated that, in the absence of the other chain, both homodimers can be formed.

To reiterate, the native frog skin secretion contained significantly more heterodimer A-B than homodimer B-B, whereas homodimer A-A was not detected.

4. Discussion

Here, we report the molecular cloning of cDNAs encoding the precursor sequences of two chains, A and B, that constitute the heterodimeric peptide, distinctin, from the skin secretion of *Phyllomedusa burmeisteri*. The corresponding mature peptide gene products were detected

in chromatographic fractions of the skin secretion as a distinctin heterodimer using MS. The only heterodimeric peptide described to date in amphibian skin secretions is the peptide, distinctin, from *Phyllomedusa distincta*, with a fully-established primary structure and antimicrobial activity [10]. The homodimer B-B, was also identified in the skin secretion of *P. burmeisteri*, and this is the first report of a naturally-occurring distinctin homodimer (Fig. 2A). These data indicate that *in vivo*, distinctin has a strong tendency to exist as a disulfide-bridged dimer. Heterodimerization seems to be preferred, but the detection of the homodimer suggests that rather than to exist as a monomer, excess peptide chains will homodimerize. Interestingly, the cloning and sequencing data indicate that the chains which *in vivo* are detected as heterodimers, are encoded by two different mRNA-encoded precursors, each with their own start and stop codon, that probably represent two distinct gene products.

Scrutinizing the protein sequence database (UniProt) found that *P. burmeisteri* chain B exhibits a 71% sequence homology with a distinctin-like peptide chain sequence previously cloned from *Phyllomedusa azurea* [24]. This sequence does not contain any cysteinyl residues and a chain A equivalent in this species was not reported. Thus, this species of phyllomedusine frog provides further, albeit indirect evidence, that chain B appears not to be encoded by the same ORF as chain A.

The mechanism behind the specific dimerization of the two *P. burmeisteri* chains from a pool of peptides containing single free cysteine residues, remains intriguing. Other natural hetero- and/or homodimeric peptides from venoms have been cloned and their nucleic acid sequences reported. These include the paralytic heterodimer, pimplin, from the wasp, *Pimpla hypochondriaca* [25]; the histamine-releasing homodimer, pilosulin 5, from the ant, *Myrmecia pilosula* [26]; hetero- and homo-dimeric αD-conotoxins (VxXXA, VxXXB and VxXXC) found in the venoms of several marine cone snails of the genus *Conus* [27]; salmorin, a thrombin-induced fibrinogen-clotting inhibitor from the snake, *Agkistrodon halys* [28]; numerous dimeric disintegrins, cloned from *Vipera* and *Echis* snakes [29-31]; irditoxin, a neurotoxic dimer from the brown treesnake, *Boiga irregularis* [32]; and various secretory enzymes of the phospholipase A2 family, such as the heterodimers, imperatoxin I and phospholipidin from *Pandinus imperator* [33, 34] –, and HDP-1P and HDP-2P subunits of the neurotoxic and anti-coagulant heterodimer from *Vipera nikolskii* [35]. As in the case of the well-known example insulin [36], most of these heterodimers arise from a single mRNA precursor, which is translated in one continuous peptide sequence. This is thought to be a crucial step in the heterodimer formation, which occurs during or immediately after translation. A connecting peptide (of varying length in insulin and the various venom

heterodimers) between both mature chains is spliced out by specific endoproteases, and this occurs after the disulfide bridge(s) formation.

The cDNAs of disintegrins, salmorin and irditoxin chains represent an exception to this rule, with both monomers encoded by different open reading frames. However, in all these cases, the pre-peptide sequences of A and B chains are very similar or almost identical (typically >70% sequence identity), which suggests that a gene duplication lies at the origin of both ORFs. In disintegrins, for example, it is suggested that the A chain precursor gene was derived from a B chain gene duplication. Subsequent deletion of a continuous and large base pair sequence of the ORF, encoding a C-terminal metalloprotease domain in the B chain pre-peptide gene and of a N-terminal portion of the disintegrin domain, are thought to produce the final structure of the A chain [30]. Therefore, the disulfide connection between the subunits would proceed similarly as in homodimers (which are also detected in these venoms), where the dimerization takes place by virtue of the simultaneous presence of the chains in the endoplasmic reticulum (ER) during the translation process.

The case of the heterodimeric *P. burmeisteri* distinctin is markedly different. The peptides representing the signal peptide, acidic spacer and mature peptide of each chain all exhibit different lengths as well as many sequence dissimilarities, yielding an overall sequence identity of only 47%.

This suggests that a different mechanism may be behind the dimerization of these dissimilar subunit chains, perhaps comparable to what happens when antibody molecules (immunoglobulins) are maturing, with heavy and light chains translated from different chromosomes “finding” each other and oxidizing in the ER [37]. This is a hypothesis, and the actual mechanism of *in vivo* heterodimer formation is still unclear.

In an attempt to obtain information on potential physicochemical constraints in the formation of the possible dimers from the two subunits identified, *in vitro* dimerization experiments were performed with synthetic replicates of the monomer peptides. However, this did not provide clues as to whether (sequence specific) physiochemical phenomena would preferably direct the formation of one dimer over the other. Our results indicate that both the heterodimer and the two homodimers can be formed *in vitro*. This is similar to what has been described for magainin and conotoxin synthetic dimers [38, 39]. It should be mentioned that during *in-vitro* oxidation experiments cystine formation could be induced by oxygen or trace amounts of metals. In addition, it is also very well possible that the synthetic peptides do not have the proper secondary/tertiary structure to promote chain specific dimerization and/or induce disulfide bond formation.

Although, in the native *P. burmeisteri* skin secretion the main compound detected was the heterodimer, followed by the homodimer B-B, no homodimer A-A, or A or B monomers, were detected. Why the heterodimer prevails in the *P. burmeisteri* skin secretion remains an unanswered question, and we can only speculate at this time. Structure modeling and NMR studies on the similar distinctin chains of *P. distincta* has indicated that matching hydrophobic areas on the outer surfaces of chain A and B and alpha-helices, may play a role in bringing both chains together [19, 40-42]. These studies, however, did not look at the structure of the homodimers.

Comparative proteolytic degradation assays of distinctin and synthetic peptide analogues reported that distinctin is more resistant to serine protease breakdown as a heterodimer than as monomers or homodimers [19, 20]. The situation in the *P. burmeisteri* skin secretion, with most of the distinctin occurring as a heterodimer, may reflect a similar situation.

Previous investigations have assessed the antimicrobial activity of all possible forms of the closely-related *P. distincta* distinctins [19, 20]. They found that the heterodimer has broad-spectrum antimicrobial activity, and that the dimers (hetero- or homo-) are more active than the separate monomers.

With the *P. burmeisteri* distinctins differing in only a single amino acid residue, the bioactivity is likely to be very similar. This would mean that the relative amounts of distinctin chains in the *P. burmeisteri* skin secretion is consistent with maximization of its bioactivity.

To assess this, we performed pilot bioactivity studies (*E. coli* growth inhibition test) using dilution series of equivalent amounts of monomeric and *in vitro* dimerized synthetic peptides (see Fig. 4). Our preliminary data indicate that indeed the *P. burmeisteri* dimers have higher specific bioactivity than the monomers, and that the sample containing the heterodimer exhibits the highest antibiotic effect, confirming what was reported for *P. distincta* distinctin.

With the present emergence of multiple-drug resistant strains of many pathogenic micro-organisms and diseases requiring a pharmaceutical solution, the development of novel and potently-active pharmaceutical agents with potential clinical and therapeutic applications could be exemplified by natural molecules like the *P. burmeisteri* distinctin heterodimer reported here.

5. Conclusion

Distinctin, a 5.4 kDa heterodimeric peptide with antimicrobial activity was identified within the skin secretion of *Phyllomedusa burmeisteri*. Molecular cloning of the cDNA encoding the two chains that constitute this heterodimeric peptide reveals they originate from two different mRNA-encoded precursors, each with their separate start and stop codon. MS analysis showed that in the crude venom the two chains are primarily present as the heterodimer A-B, a subfraction as homodimer B-B, whereas neither homodimer AA nor the individual A of B chains were observed. *In vitro* oxidation experiments with synthetic chains showed no preferential formation of hetero- of homodimer, suggesting there is an alternative mechanism for dimer formation *in-vivo*. To our knowledge this is the first example of an amphibian skin dimeric peptide that is formed by covalent linkage of two chains that are the products of different mRNAs.

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Figures Legends

Figure 1: Nucleotide sequences of precursor cDNAs encoding *Phyllomedusa burmeisteri* distinctin chain A (A) and distinctin chain B (B) cloned from a skin secretion-derived library. Putative signal peptides (double-underlined), mature processed peptides (single-underlined) and stop codons (asterisks) are indicated.

Figure 2: Distinctin dimers in native *P. burmeisteri* skin secretion. (A) Combined MS spectrum of elution times in nanoLC chromatogram of both distinctin dimers. Spectrum shows presence of heterodimer (green peaks) and homodimer B-B (magenta peaks), among several other (higher and lower abundant) non-distinctin peptide peaks. Both distinctin dimers appear as multiply charged ions (z=5 until z=10) with heterodimer being most abundant. (B) Tandem MS of heterodimer (precursor ion at m/z 909.190 selected ($[M+6H]^{6+}$)) showing b-, y-, and i- (immonium) ions from both chains A and B. Insert shows primary structure of chain A and B with all detected b- and y- ions indicated.

Figure 3: Zoomed-in area of PTM-driven differential peptide display of native crude *P. burmeisteri* venom (in blue) and DTT-reduced venom (in red): inserts show $[M+4H]^{4+}$ ions of distinctin monomeric chains A (A) and B (B) and $[M+7H]^{7+}$ ions of distinctin heterodimer (AB). Note that sum of mass (A) and mass (B) minus mass (2H) exactly equals mass (AB). Ions common in both samples have blue and red colors superimposed, yielding blackish spots. Red color of monomers A and B indicate that these ions are not observed in native venom, whereas, conversely, blue heterodimer ions reveal that no heterodimer was detected in DTT-reduced venom. (Full PTM-driven differential peptide display is provided as Supplementary Fig. 1).

Figure 4: Deconvoluted MS spectra of *in vitro* dimerization of synthetic distinctin chains A and B. (A-C) "SYNTHETIC CHAIN(S) A/B/A&B" represent the mixture after one week of incubation of the respective chains in water. (D-F) "SYNTHETIC DIMERS A-A/B-B/A-B" show the oxidation products after one week in bicarbonate buffer pH 8.0. Masses of respective chains (monomers) and dimers are indicated by arrows.

Supplementary Figure 1: Full PTM-driven differential peptide display of *P. burmeisteri* crude venom. Selected area is zoomed-in in Fig. 3.

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Figure 1

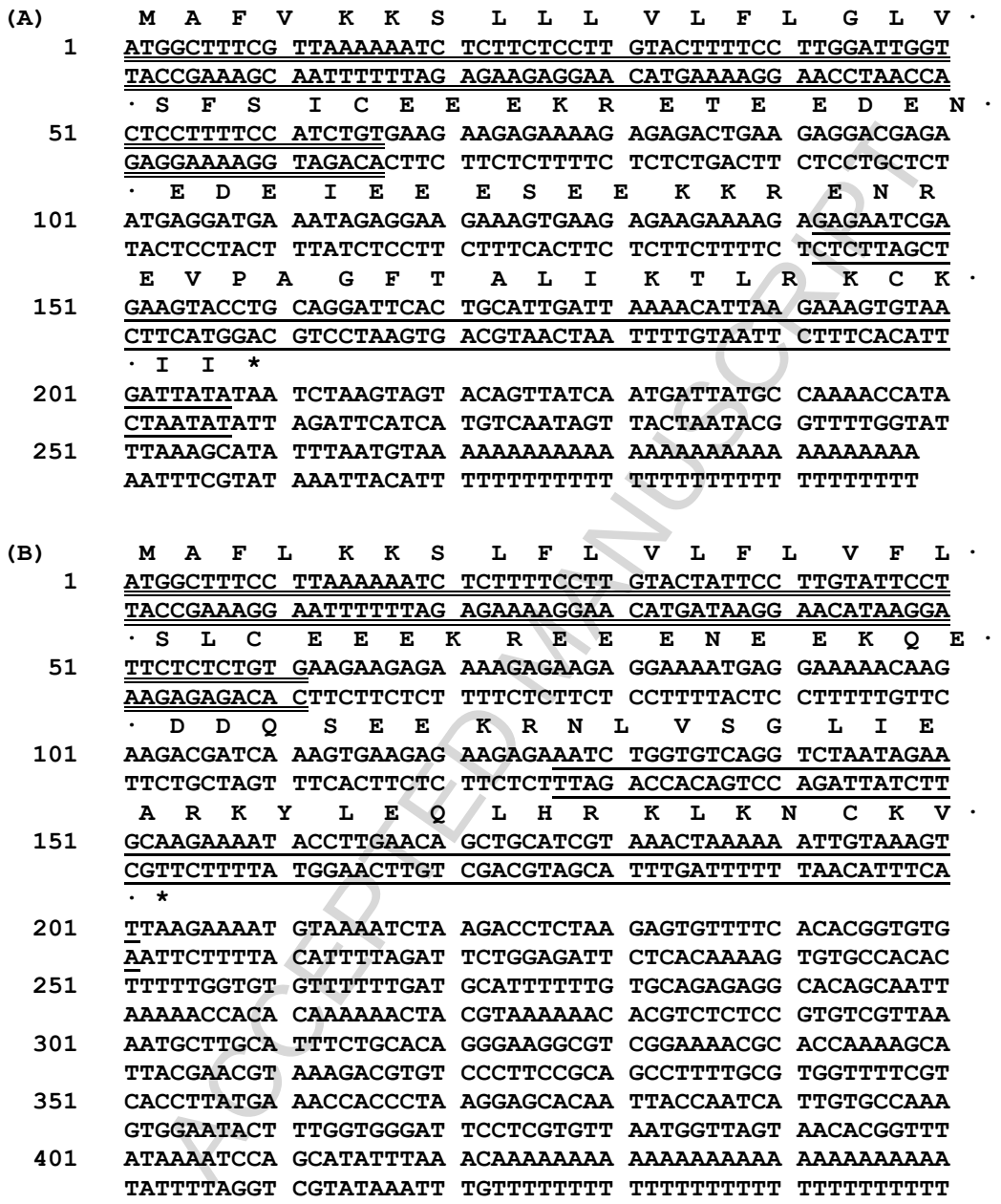


Figure 2

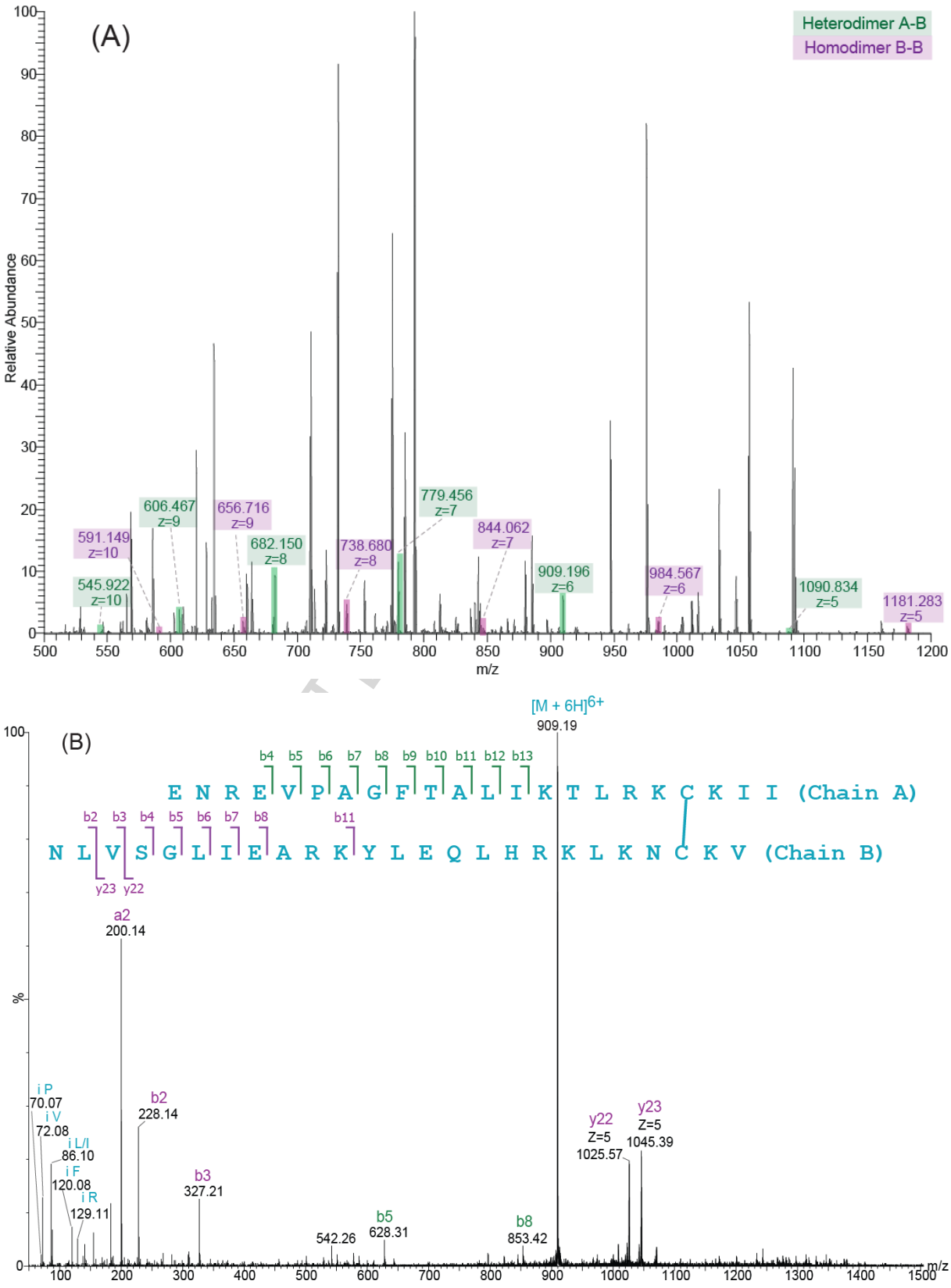


Figure 3

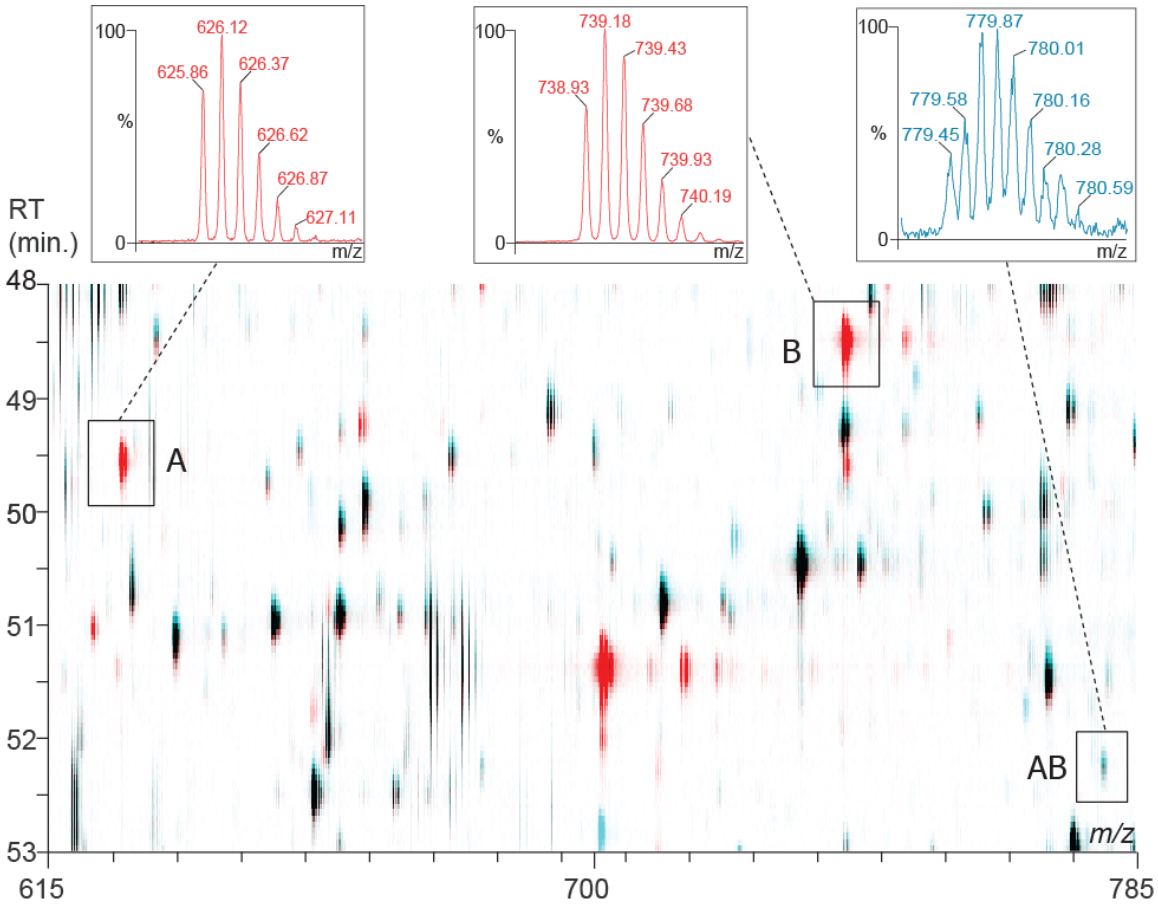
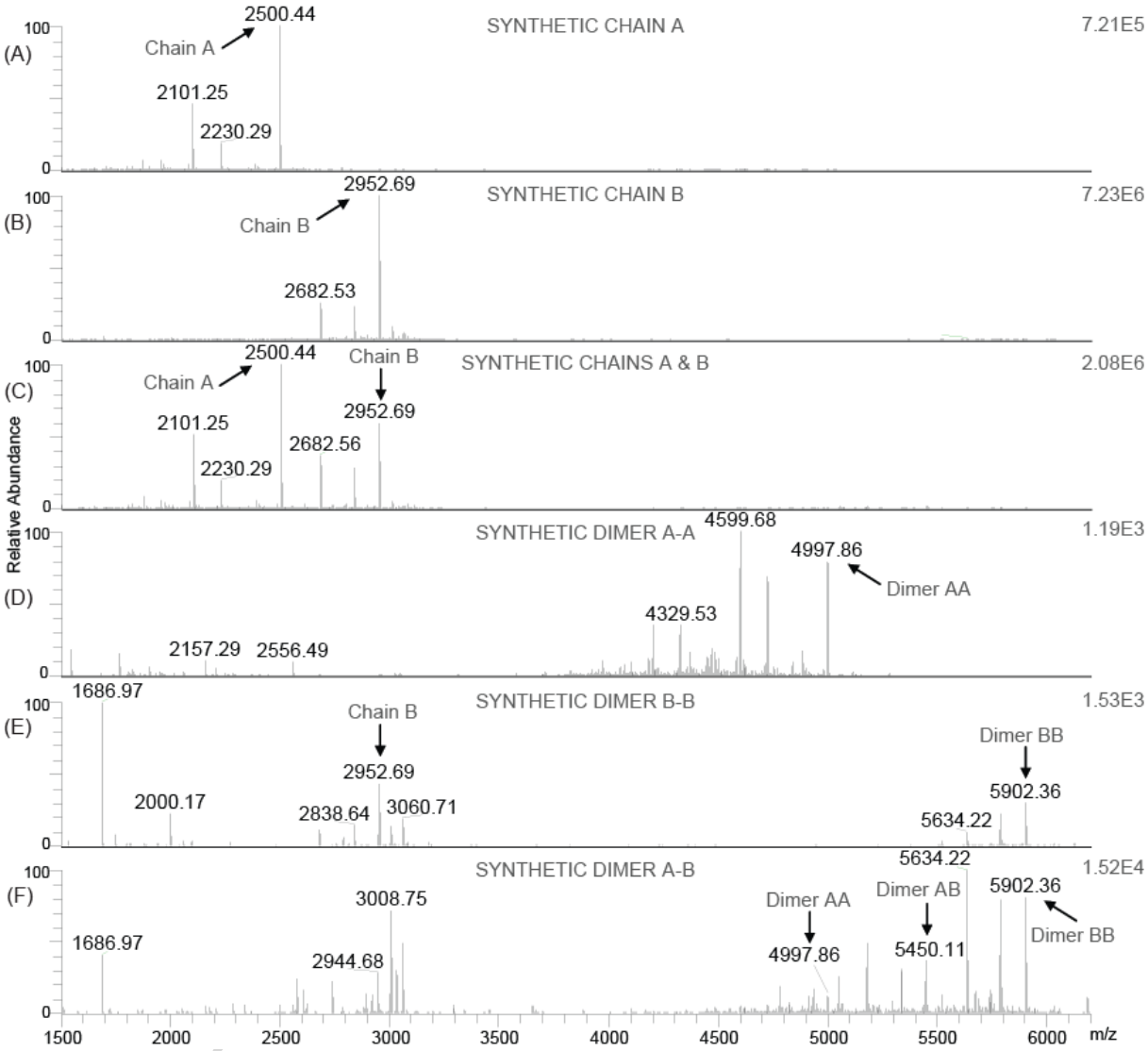
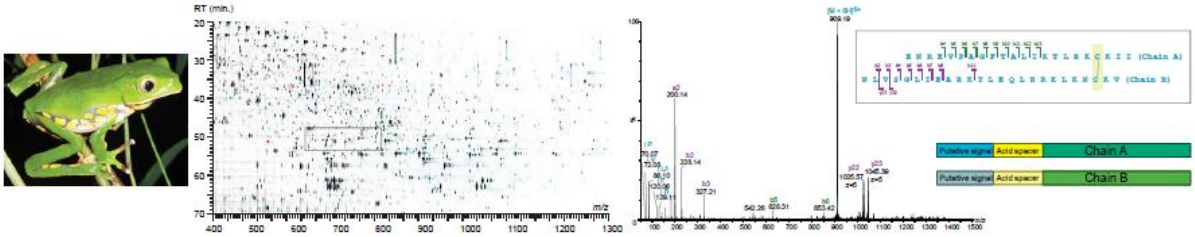


Figure 4



Graphical Abstract



Highlights

1. Peptidome/transcriptome analysis of skin secretion of *Phyllomedusa burmeisteri*.

2. The two chains of distinctin, a 5.4 kDa dimeric skin peptide, were shotgun cloned.

3. Chains of this heterodimeric peptide are encoded by two different ORFs.

4. *In vivo*, the hetero-dimeric distinctin is more abundant than homo-dimeric distinctin.

5. *In vitro* oxidation of synthetic peptides showed no preferential hetero-dimerization.