Amino Acids (2012) 43:309–316
DOI 10.1007/s00726-011-1079-8

ORIGINAL ARTICLE

Bi-functional peptides with both trypsin-inhibitory and antimicrobial activities are frequent defensive molecules in *Ranidae* amphibian skins

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Received: 15 June 2011 / Accepted: 6 September 2011 / Published online: 17 September 2011

Abstract Amphibian skins act as the first line against noxious aggression by microorganisms, parasites, and predators. Anti-microorganism activity is an important task of amphibian skins. A large amount of gene-encoded antimicrobial peptides (AMPs) has been identified from amphibian skins. Only a few of small protease inhibitors have been found in amphibian skins. From skin secretions of 5 species (*Odorrana livida*, *Hylarana nigrovittata*, *Limnonectes kuhlii, Odorrana grahami,* and *Amolops loloensis*) of Ranidae frogs, 16 small serine protease inhibitor peptides have been purified and characterized. They have lengths of 17–20 amino acid residues (aa). All of them are encoded by precursors with length of 65–70 aa. These small peptides show strong trypsin-inhibitory abilities. Some of them can exert antimicrobial activities. They share the conserved GCWTKSXXPKPC fragment in their primary structures, suggesting they belong to the same families of peptide. Signal peptides of precursors encoding these serine protease inhibitors share obvious sequence similarity with those of precursors encoding AMPs from Ranidae frogs. The current results suggest that these small serine protease inhibitors are the common defensive compounds in frog skin of Ranidae as amphibian skin AMPs.

Keywords Amphibian Skin · Antimicrobial peptide · Innate immunity · Protease inhibitor · Serine

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Electronic supplementary material The online version of this article (doi:10.1007/s00726-011-1079-8) contains supplementary material, which is available to authorized users.

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Introduction

Amphibian skins play an important role in the survival of amphibians and help them adapt to a wide range of habitats and ecological conditions (Zasloff 2002). Therefore, they are endowed with an excellent defense system which comprises encoding antimicrobial peptides (Conlon et al. 2004; Brogden 2005). These peptides have pharmacological effects including cardiotoxic, myotoxic, neurotoxic, and antimicrobial activities (Clarke 1997; Li et al. 2007b; Montecucchi et al. 1981; Liu et al. 2010; You et al. 2009; Yang et al. 2009) All these properties clearly adversely affect a potential predators or pathogens.

Serine protease inhibitors with molecular weights larger than 5 kDa are found widely in animals, plants and microorganisms (Bode and Huber 1992; Christeller 2005; Li et al. 2008). Many pathogens are known to produce extracellular proteases, which play important roles in the development of diseases (Christeller 2005). Protease inhibitors play an important role in defending against microorganism or pest by combating their proteases (Christeller 2005). Recently, we have identified a few small

serine protease inhibitors with molecular mass less than 3 kDa from amphibian skins of *O. grahami* (Li et al. 2007b; Li et al. 2008; Li et al. 2007a), which belong to the peptide family of Ranacyclin (Ma et al. 2010). One of them (Ranacyclin-B1) had both trypsin-inhibitory and antimicrobial activities (Li et al. 2007a). All of them have similar signal peptide sequences with those of AMPs found in skins of Ranidae frogs. Those results suggest that small trypsin inhibitors play defensive roles by exerting trypsin-inhibition or/and microorganism-killing.

In order to investigate if there is similar family of trypsin inhibitor with small molecular weight as Ranacyclin-B1 in Ranidae frogs' skin, skin secretions collected from five Ranidae species (*O. livida*, *H. nigrovittata*, *L. kuhlii, O. grahami,* and *A. loloensis*) were used to purify possible trypsin inhibitors. Their primary structures were determined by Edman degradation and cDNA cloning; their trypsin-inhibitory and antimicrobial activities were tested. The possible evolutionary relationship between amphibian AMPs and small protease inhibitors was discussed.

Materials and methods

Collection of frog skin secretions

Adult specimens of five amphibians (*O. livida*, *H. nigrovittata*, *L. kuhlii, O. grahami,* and *A. loloensis*) of both sexes ($n = 20$; weight range 20–40 g) were collected in Yunnan Province of China. Skin secretions were collected according to our previous reports (Liu et al. 2010; Li et al. 2007a, b; Wang et al. 2008; Mangoni et al. 2003). In brief, animals were put into a cylinder container containing a piece of absorbent cotton saturated with anhydrous ether. On exposing to anhydrous ether for 1–2 min, secretions were seen to exude from their skins. Skin secretions were collected by washing each frog with 0.1 M NaCl solution (containing protease inhibitor cocktail, Sigma). The collected solutions (100–150 ml of total volume) were quickly centrifuged and the supernatants were lyophilized. Lyophilized skin secretions were kept at −20°C till use. All the experiments were approved by Kunming Institute of Zoology, Chinese Academy of Sciences.

Peptide purification

Lyophilized skin secretion samples (200–300 mg, total absorbance at 280 nm is 300–500) was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0 (PBS) and applied on a Sephadex G-50 gel filtration column (2.6 cm diameter, 100 cm length, Amersham Biosciences) equilibrated with the same PBS buffer. Elution was performed with the same buffer, collecting fractions of 3.0 ml per 10 min. The absorbance of the eluate was monitored at 280 nm. Every fraction was subjected to trypsin inhibition testing as described below. The eluted protein peaks, which show trypsin-inhibitory activity, were pooled and purified further by a C_{18} reversed-phase high performance liquid chromatography (RP-HPLC, Hypersil BDS C_{18}, 25 × 0.46 cm) column equilibrated with 0.1% (v/v) trifluoroacetic acid/water with the elution profile as indicated at a flow rate of 0.7 ml/min. The absorbance of the eluate was monitored at 280 and 220 nm. Sephadex G-50 gel filtration and C_{18} RP-HPLC of *O. livida*, *H. nigrovittata* and *A. loloensis* have reported in our previous works to purify AMPs (Liu et al. 2010; Ma et al. 2010; Wang et al. 2008). The protein peak III from Fig. 1d was pooled (27 ml), lyophilized, and re-suspended in 3 ml 0.1 M PBS, and purified further by a C_{18} RP-HPLC column. All the purified interesting peptides from aliquots were pooled and subjected to further study.

Mass spectrometry

Mass fingerprints (MFPs) were obtained by using a Voyager-DETM Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI-TOF-MS, Voyager DE Pro, Applied Biosystems) in positive ion and linear mode with operating parameters as follows: the ion acceleration voltage was 20 kV, the accumulating time of single scanning was 50 s, polypeptide mass standard (Applied Biosystems) serving as external standard. The accuracy of mass determinations was within 0.1%.

Amino acid sequencing

Complete amino acid sequences of small trypsin inhibitors purified from these frog skin secretions were determined by the automated Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491.

cDNA library construction

As our previous reports (Li et al. 2007a, b), SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA) was used to synthesize cDNA, using frog skin total RNA extracted by TRIzol (Life Technologies, Ltd.) as template. The first strand was synthesized by using cDNA 3′ SMART CDS Primer II A, 5′-AAGCAGTGGTATCAACGCA GAGTACT (30) N-1 N-3′ (N = A, C, G or T; N-1 = A, G or C), and SMART II An oligonucleotide, 5′-AAGC AGTGGTATCAACGCAGAGTACGCGGG-3′. The second strand was amplified using Advantage polymerase by 5′ PCR primer II A, 5′-AAGCAGTGGTATCAACGCA GAGT-3′. Using these synthesized cDNA, five skin cDNA libraries of these frogs were constructed as our previous

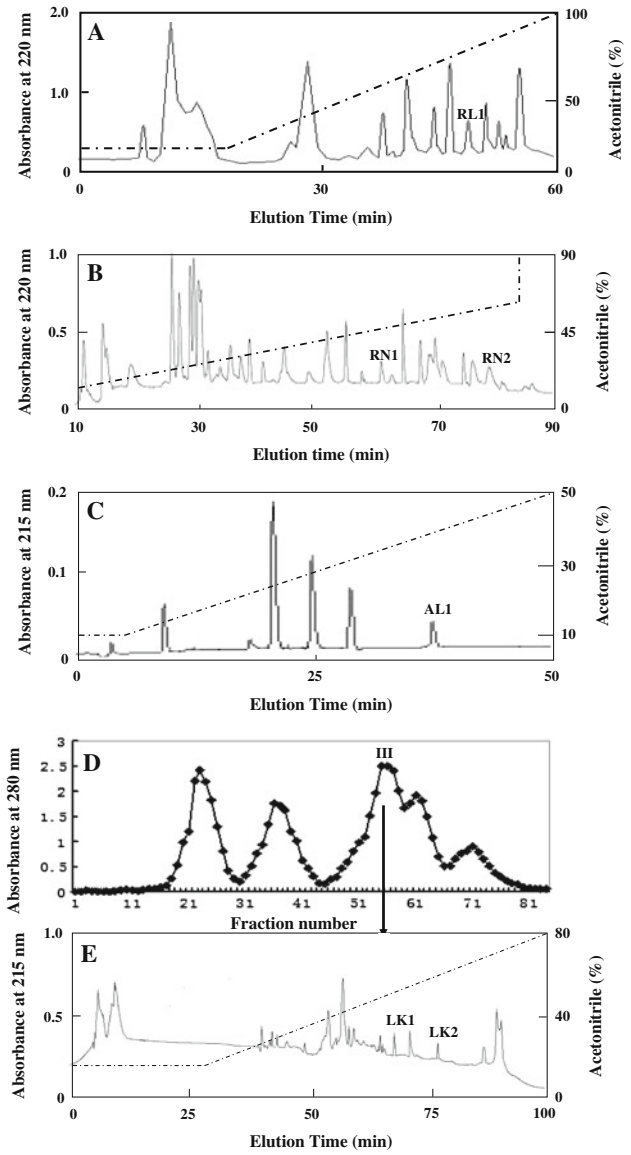

Fig. 1 Purification of Ranacyclin-B-like peptides from skin secretions of Ranidae species. **a–c** HPLC separation. The protein peak III from Sephadex G-50 gel filtration (**d**) was purified further by RP-HPLC on a C18 column (**e**). The range of acetonitrile of the gradient was indicated by *dotted line*. For details, refer to the "Materials and methods"

reports (Li et al. 2007b; Liu et al. 2010; Ma et al. 2010; Wang et al. 2008).

cDNA cloning

According to amino acid sequences determined by Edman degradation, several specific primers were designed (Supplementary Table S1). A PCR-based method for high stringency screening of DNA libraries was used for screening and isolating the clones. Specific designed primer in the sense direction and primer II A as mentioned in "cDNA library construction" in the antisense direction were used in PCR reactions. The DNA polymerase was Advantage polymerase from Clontech (Palo Alto, CA, USA). The PCR conditions were 2 min at 94°C, followed by 30 cycles of 10 s at 92°C, 30 s at 50°C, 40 s at 72°C. DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Trypsin-inhibitory testing

The inhibition effect of test sample on the hydrolysis of synthetic chromogenic substrate N-Benzoyl-L-arginine-4-nitroanilide-hydrochloride (B-3133, Sigma) by trypsin (sigma) was assayed in 50 mM Tris–HCl, pH 7.8 at room temperature. Different amounts of test sample (final concentrations ranging from 0.01 to 10 μM) and trypsin (final concentration 10 nM) were pre-incubated for 10 min at room temperature. Substrate L-BAPNA (final concentration of 0.5 mM) was added into the mixture to initiate the reaction. The formation of *p*-nitroaniline was monitored continuously at 405 nm for 2 min. The inhibition constant *Ki* was determined according to the reported method (Li et al. 2007a, 2008).

Antimicrobial assay

Several microorganisms including *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), Gram-negative bacteria *Escherichia coli* (ATCC 25922), and fungus *Candida albicans* (ATCC 20032) were obtained from Kunming Medical College for antimicrobial assays. They were first grown in Luria–Bertani (LB) broth or yeast extract-peptone-dextrose broth, minimal inhibitory concentration (MIC) of tested sample against these microorganisms was determined as our previous described (Li et al. 2007b). The MIC is defined as the lowest concentration of test sample inhibiting microorganism growth.

Peptide synthesis

All of the peptides used in this work were synthesized by GL Biochem (Shanghai) Ltd. (Shanghai, China) and analyzed by HPLC and mass spectrometry to confirm their purity higher than 98%.

Results

Purification and characterization of small trypsin inhibitors from frog skin secretions

From frog skin secretions of *O. grahami*, we have purified and characterized two small trypsin inhibitors belonging to

the Ranacyclin B peptide family (Li et al. 2007a, b). By Sephadex G-50 gel filtration combined with C_{18} RP-HPLC, six small trypsin inhibitors were purified from 4 species of these Ranidae frogs including *O. livida*, *H. nigrovittata*, *L. kuhlii*, and *A. loloensis*, respectively, as illustrated in Fig. 1. They are named Ranacyclin-B-RL1 (from *O. livida*, Fig. 1a), Ranacyclin-B-RN1 and 2 (from *H. nigrovittata*, Fig. 1b), Ranacyclin-B-AL1 (from *A. loloensis*, Fig. 1c), and Ranacyclin-B-LK1 and -LK2 (from *L. kuhlii*, Fig. 1d, e), respectively. All of these purified trypsin inhibitors were subjected to amino acid sequencing and MALDI-TOF-MS analysis. Their amino acid sequences and mass fingerprints are illustrated in Figs. 2 and 1s, respectively. They have a predicted molecular weight of 2,164.6, 2,095.5, 1,966.3, 1,940.3, 2,175.6, and 2,118.5, respectively. The mass spectrometry analysis gave an observed mass of 2,165.0, 2,095.6, 1,966.0, 1,940.3, 2,176.0, and 2,119.0, respectively (Fig. 1s), which matched well with predicted molecular weights.

cDNA cloning

Using those degenerated primers, 30 cDNAs encoding 16 different mature short trypsin inhibitors were cloned and isolated (Fig. 2). Among them, six amino acid sequences deduced from cDNAs are identical to those amino acid sequences (Ranacyclin-B-RL1, Ranacyclin-B-RN1, Ranacyclin-B-RN2, Ranacyclin-B-LK1, Ranacyclin-B-LK2, and Ranacyclin-B-AL1) determined by Edman degradation. It further confirmed the results of Edman degradation. All these 16 sequences share high sequence similarity. Especially, there is the conserved GCWTKSXXPKPC motif in their primary structures as illustrated in Fig. 2. They belong to the same peptide family of Ranacyclin-B, which are bi-functional peptides with trypsin-inhibitory and antimicrobial activities found in the skin secretions of *O. grahami* (Li et al. 2007a, b). All of them are cationic peptides. There are multiple basic aa (Arg and/or Lys), no acidic aa in their sequences. Most of them are composed of 19 or 20 aa. Ranacyclin-B-AL1 has only 17 aa; Ranacyclin-B-RN3 has a length of 43 aa, which is extended from the original length of 19 or 20 aa due to the backward movement of the termination codon.

Trypsin inhibition

These peptides from the five species of Ranidae frogs were subjected to trypsin-inhibitory testing as listed in Table 1. All of them showed abilities to inhibit trypsin's hydrolysis against L-BAPNA. Ranacyclin B3, -B5, and -B-RL1 had similar trypsin-inhibitory abilities with a *Ki* order of 10^{-8} M. Ranacyclin-B-RN1, -B-RN2, -B-RN6, -B-LK1, and -B-LK2 had a *Ki* order of 10^{-6} M. Ranacyclin-B-AL1 had the weakest trypsin-inhibitory activity with a *Ki* of 10^{-4} M.

Antimicrobial activity

All of these Ranacyclin B-like peptides showed antimicrobial activities as listed in Table 1. At concentrations of 5.8–51.5 μM, they could kill *S. aureus*. Ranacyclin-B-RL1 showed the strongest antimicrobial activity. It shows ability

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Ranacyclin B1      AALKGCWTKSIPPKPCFGKR (Li.2007)
Ranacyclin B2      AALKGCWTKSIPPKPCFRKR (Li.2007)
Ranacyclin B3      AALKGCWTKSIPPKPCSGKR (EU642568)
Ranacyclin B4      AALKGCWTNSIPPKPCSGKR (EU642569)
Ranacyclin B5      AALRGCWTKSIPPKPCSGKR (EU352861)
Ranacyclin-B-RL1   AALRGCWTKSIPPKPCPGKR (EU294106)
Ranacyclin-B-RN1   SALVGCWTKSYPPKPCFGR  (EU136423)
Ranacyclin-B-RN2   SALVGCGTKSYPPKPCFGR  (EU136414)
Ranacyclin-B-RN3   SALVGCWTKSYPPKPVSVDDKTCLANHLMWNIIWLNARCLMKK (EU136415)
Ranacyclin-B-RN4   SALVGCWTKSYPPKPCFGRG (EU136416)
Ranacyclin-B-RN5   SALVGCWTKSYPPKPCIGRG (EU136424)
Ranacyclin-B-RN6   SALVGCWTKSYPPNPCFGRG (EU136425)
Ranacyclin-B-LK1   SALVGCWTKSWPPKPCFGRG (EU346895)
Ranacyclin-B-LK2   SALVGCWTKSWPPKPCFGR  (EU346896)
Ranacyclin-B-LK3   SMLVGCWTKSYPPKPCFGRG (EU346897)
Ranacyclin-B-AL1   AAFRGCWTKNYSPKPCL    (EU311545)
pLR                 LVRGCWTKSYPPKPCFVRG (Salmon.2001)
pYR                 YLKGCWTKSYPPKPCFSR  (Graham.2001)
SFTI-1                GRC-TKSIPPI-CFPD  (Hernandez.2000;Korsinczky.2001)
BBI                    -CTP1SXPP-QC-    (Qi.2005)
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Fig. 2 Sequence comparison of Ranacyclin-B-like peptides (including Genebank number), Bowman–Birk inhibitors reactive loop (BBI), and SFTI-1. The bar (−) was introduced for the optimal alignment

Table 1 Trypsin-inhibitory and antimicrobial activities of amphibian skin peptides

Peptides	Trypsin-inhibitory, *Ki* values (μM)	Antimicrobial (MIC, μM)			
		C. albicans albicans albicans valbicans albicans	*S. aureus*	*E. coli*	*B. subtilis*
Ranacyclin B3	0.026	23.4	11.7	NA	NA
Ranacyclin B5	0.099	23.1	5.8	NA	NA
Ranacyclin-B-RL1	0.099	46.2	5.8	46.2	46.2
Ranacyclin-B-RN1	2.438	NA	6.0	NA	NA
Ranacyclin-B-RN2	5.350	NA	12.7	NA	NA
Ranacyclin-B-RN6	3.417	46.7	11.7	NA	NA
Ranacyclin-B-LK1	3.106	NA	23.0	NA	NA
Ranacyclin-B-LK2	2.818	NA	11.8	23.6	NA
Ranacyclin-B-AL1	115.118	NA	51.5	NA	25.8

Ki inhibitory constant, *MIC* minimal peptide concentration required for total inhibition of cell growth in liquid medium. These concentrations represent mean values of three independent experiments performed in duplicates

to kill these four tested microorganism strains. Four of these peptides have antimicrobial activities against *C. albicans* (Ranacyclin B3, -B5, -RL1, and -RN6). Most of these Ranacyclin peptides had no effects on *E. coli* and *B. subtilis*. High concentrations of Ranacyclin-RL2, -LK2, or -AL1) showed antimicrobial activities against these two microorganisms.

Precursor sequence comparison between amphibian AMPs and short protease inhibitors

Multiple AMP families including palustrin, esculentin 1, esculentin 2, ranatuerin, brevinin 1, brevinin 2, temporin, nigrocin, and gaegurin have been identified from Ranidae frog skins. Two peptides (Ranacyclin T, HV-BBI) with high sequence similarity to Ranacyclin B was also found in skin secretions of *R. esculenta* and *R. versabilis*, respectively (Ma et al. 2010; Song et al. 2008). All of these AMPs and short trypsin inhibitors were aligned as illustrated in Fig. 3. They share highly conserved signal peptide sequences. In addition, they have the same di-basic enzymatic processing sites (-KR- or -RR-), suggesting that they share the same enzymatic processing pathway.

Discussion

In this work, six bi-functional peptides were purified and characterized from four species of frogs belonging to Ranidae family, which are *O. livida*, *H. nigrovittata*, *L. kuhlii*, and *A. loloensis*, respectively. They belong to the peptide family of Ranacyclin, which is originally found in the skin secretions of *R. pipiens* (Salmon et al. 2001) and first named by Mangoni et al. (2003). By cDNA cloning, 16 Ranacyclin B-like peptides were identified from these Ranidae amphibian skins (Fig. 2). They are highly conserved and are found to be composed of 63 aa, including an N-terminal signal peptide, followed by an acidic spacer peptide and a C-terminal mature peptide. The mature peptide is similar to SFTI-1, which is a most potent known naturally occurring Bowman–Birk inhibitor (Hernandez et al. 2000; Korsinczky et al. 2001) (Fig. 2). Our previous work has indicated that Ranacyclin-B is a member of Bowman–Birk inhibitors family (Li et al. 2007a). It adopts two antiparallel β-strands conformation with a disulphide bond forming between P4 Cys and P7' Cys. This disulphide bond results in a reactive loop which is similar with but not exactly the same as that in SFTI-1 (Li et al. 2007a). Compared with the conserved nine-residue inhibition reactive loop (CTP1SXPPQC, which P1 represents the residue determining the inhibitor specificity) in other Bowman–Birk inhibitors (Prakash et al. 1996; Qi et al. 2005; Fig. 2), there are special 11-residue loop (CWTK(P1)SXXPKPC, the P1 residue is Lys) in these Ranacyclin B-like peptides, with two more residues of ^{P3}Trp and $^{P6'}$Pro. The sequence and the conformation of residues ^{P2}TKSIPP$^{P4'}$ are the same as those of Bowman–Birk inhibitor like SFTI-1, whereas two cysteines in our peptides forming the disulfide bond are at P4 and P7′ positions, different from the P3 and P6′ positions in SFTI-1. The disulfide-forming positions create a special 11-residue loop instead of the conserved 9-residue one in other Bowman–Birk inhibitors. All of these Ranacyclin B-like peptides have only one P1 residue, lysine in their inhibition reactive loop, indicating that they have the same specificity of inhibition.

All of them showed strong inhibitory activity against trypsin except Ranacyclin-B-AL1. Inhibitory constants of Ranacyclin B3, -B5, and -B-RL1 against trypsin are in an order of 10^{-8} M while those of Ranacyclin-B-RN1, -B-RN2, -B-RN6, -B-LK1, and -B-LK2 are in an order

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Ranacyclin B4          MFTLKKSLFVLFFLGIVSLSVCEHNRDADEEDGGE--------AIGGEVKRAALKGCW------ 50
Ranacyclin-B-RL1       MFTMKKSLLFLFFLGIVSLSFCEQERDADEEDGGR--------VTEEEVKRAALRGCW------ 50
Ranacyclin-B-RN1       MFTMKKSLLLLFFLGIVSLSLCGQERDADEEDGGE--------VTVEEVKRSALVGCW------ 50
Ranacyclin-B-LK1       MFTMKKSLLLLFFLGIVSLSLCGQERDADEEDGGE--------VTEEEVKRSALVGCW------ 50
Ranacyclin T           MFTMKKTLLVLFFLGVVSLSLCVEERDADEEDGGE--------VMEEEVKRGALRGCW------ 50
Ranacyclin-B-AL1       MFTMKKSLLLLFFLGAISLSLCEQERDADEEDGGE--------VTEEEVKRAAFRGCW------ 50
HV-BBI                 MFTLKKSLLLLFFLGTISLSLCEQERDSDDEDQGE--------VTEQVVKR-SVIGCW------ 49
Palustrin 1c           MFTMKKSLLLLFFLGTISLSLCEEERGADEEEGD----------GEKLTKRALSILR------- 47
Esculentin 1E          MFTLKKSMLLLFFLGTINLSLCEEERDADEEERRD----NPDESEVEVEKR-FLPLLA------ 53
Esculentin 2Vb         MFTMKKSLLLLFFLGTISLSLCEEERGADEEEGDG----------EKLMKRGLFSILKGVGKIA 53
Ranatuerin 2p          MFTMKKSLLLFFFLGTISLSLCEQERGADEDDGVE--------ITEEEVKRGLMDTVKNV---- 52
Brevinin-1PLb          MFTTKKSMLLLFFLGTINLSLCEEERNAEEERRDEP-----DEMNVEVEKR-FLPLIAGL---- 54
Brevinin-2Ef           MFTMKKSLLLIFFLGTISLSLCQEERNADDDD-GE---------MTEEEKRGIMDTLKNLAKTA 54
Gaegurin-4             MFTMKKSLLFLFFLGTISLSLCEEERSADEDDGGE--------MTEEEVKRGILDTLKQFAKGV 56
Temporin-LTf           MFTMKKSLLLLFFLGTINLSLCEEERNA-EEERRDDD----DEGGAEVQKR-FLPIALKA---- 54
Nigrocin-2             MFTTKKSILLLFFLGMVSLALCEQERDANEEERRDEL----DERDVEAIKRGLLSKVLGV---- 56
                       ***.***::.:**** :.*:.* .:*.* :::          :*

Ranacyclin B4          TKSIP---------PKPCSGKR-- 63
Ranacyclin-B-RL1       TKSIP---------PKPCPGKR-- 63
Ranacyclin-B-RN1       TKSYP---------PKPCFGRG-- 63
Ranacyclin-B-LK1       TKSWP---------PKPCFGRG-- 63
Ranacyclin T           TKSYP---------PKPCK----- 60
Odorranain-B-AL1       TKNYS---------PKPCLGKR-- 63
HV-BBI                 TKSIP---------PRPCFVKG-- 62
Palustrin 1c           GLEK---LAKMGIALTNCKATKKC 68
Esculentin 1E          GLAA------NFLPKIFCKITRKC 71
Esculentin 2Vb         IKGLGKNLGKMGLDLVSCKISKEC 77
Ranatuerin 2p          AKNLAG----HMLDKLKCKITGC- 71
Brevinin-1PLb          AANFL--------PKIFCAITKKC 70
Brevinin-2Ef           GKGALQS----LVKMASCKLSGQC 74
Gaegurin-4             GKDLVKGAAQGVLSTVSCKLAKTC 80
Temporin-LTf           LGSIF--------PKILGK----- 65
Nigrocin-2             G----------KKVLCGVSGLC 68
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Fig. 3 Precursor sequence comparison of Ranacyclin-B-like peptides with AMPs from Ranidae amphibians. The *bar* (−) was introduced for the optimal alignment. The predicted signal peptides are in italic. The predicted enzymatic processing sites are *boxed*. Sequences of Ranacyclin-B-like peptides (this report) Ranacyclin T (Ma et al. 2010), HV-BBI (Song et al. 2008), Palustrin 1c (Chen et al. 2006), Brevinin 1E (Simmaco et al. 1994), Esculentin 2Vb (Chen et al. 2006), Ranatuerin 2p (Chen et al. 2003), Brevinin-1PLb (Zhou et al. 2007), Brevinin-2Ef (Simmaco et al. 1994), Temporin LTf (Wang et al. 2009), Nigrocin-2 (Wang et al. 2010) and Gaegurin-4 (Wang et al. 2009) are used for the alignment

of 10^{-6} M. The most significant difference in these two groups of Ranacyclin-B-like peptides is that there is a Phe after the P7' Cys in Ranacyclin-B-RN1, -B-RN2, -B-RN6, -B-LK1, and -B-LK2 but not in Ranacyclin B3, -B5, and -B-RL1. Compared with other Ranacyclin B-like peptides with 19 or 20 aa, Ranacyclin-B-AL1 has the shortest length with only 17 aa. There is a CWTKNYSPKPC reactive loop in Ranacyclin-B-AL1 compared to the reactive loops of CWTKSXPPKPC (where X is Ile, Tyr, or Trp) in most of Ranacyclin-B-like peptides. Interestingly, the presence Ile in position between P1′ and P3′ appears to give stronger inhibitory activity than Tyr or Trp. There are two obvious different residues in positions of P1′ and P3′ (Asn/Ser and Ser/Pro). In addition, there is an absence of 2–3 aa fragment at the C-terminus of Ranacyclin-B-AL1. Probably, these structural differences contribute their functional differences. Much more work is necessary to study the structure-function relationship in this family of peptides.

In addition to their trypsin-inhibitory activities, these Ranacyclin-B-like peptides have also been found to contain antimicrobial abilities (Table 1). All of them have antimicrobial activities against *S. aureus*. Some of them have broad antimicrobial spectrum, such as Ranacyclin-B-RL1, which showed antimicrobial abilities against all the tested microorganisms. Most of them showed narrow antimicrobial spectrum. Some of these antimicrobial peptides show significantly different antimicrobial activities although they share high sequence similarity. For example, Ranacyclin-B-LK1 (SALVGCWTKSWPPKPCFGRG) and Ranacyclin-B-LK2 (SALVGCWTKSWPPKPCFGR) have only one amino acid difference (the last Gly of LK1) but they have obviously different functions. This is because of more positive net charge than Ranacyclin-B-LK1, Ranacyclin-B-LK2 shows moderate antimicrobial abilities against *E. coli*, *S. aureus*, but Ranacyclin-B-LK1 only shows antimicrobial activity against *S. aureus*. These results indicate that

Ranacyclin-B-like peptides are bi-functional molecules with both trypsin-inhibitory and antimicrobial activities.

Aside from the mature peptide region, the overall structure of Ranacyclin-B-like peptide precursors is quite similar to precursors of AMP families from Ranidae amphibians, such as peptide families of palustrin, esculentin 1, esculentin 2, ranatuerin, brevinin-1, brevinin-2, gaegurin, temporin, nigrocin (Chen et al. 2003; Chen et al. 2006; Park et al. 1994; Simmaco et al. 1994; Wang et al. 2009, 2010; Zhou et al. 2007; Fig. 3), suggesting that they have the same ancestor. AMPs from Ranidae amphibians have been suggested to originate from a common ancestor (Conlon et al. 2004). Based on the current study, we propose that AMPs from Ranidae amphibians and Ranacyclin-B-like peptides share a common ancestor. Functionally, both AMPs and trypsin inhibitors are direct defensive molecules against microorganisms or pests. The functional similarity also implies their evolutionary connection.

Up to now, Ranacyclin-B-like peptides have been identified from at least nine Ranidae species of amphibians including *O. livida* (Ranacyclin-B-RL1), *H. nigrovittata* (Ranacyclin-B-RN1-5), *L. kuhlii* (Ranacyclin-B-RK1-3), *O. grahami* (Ranacyclin B1-5), *A. loloensis* (Ranacyclin-B-AL1), *O. versabilis* (HV-BBI) (Song et al. 2008), *R. esculenta* (Ranacyclins) (Mangoni et al. 2003), *R. pipiens* [Peptide leucine arginine (pLR)] (Salmon et al. 2001), and *R. sevosa* [peptide tyrosine arginine (pYR)] (Graham et al. 2005). pLR and pYR were originally identified as mammalian immune system regulatory peptides (Salmon et al. 2001; Graham et al. 2005). Ranacyclins from *R. esculenta* (Ma et al. 2010) were originally identified as antimicrobial peptides. Their effects on trypsin were not assayed. Based on sequence alignment we can see that the similarities among these Ranacyclin-B-like peptides are quite high (Fig. 2). Most of them are bi-functional peptides with both trypsin-inhibitory and antimicrobial activities. These results suggest that this type of bi-functional peptide family is common defensive molecules in Ranidae amphibian skins although more work is necessary to identify Ranacyclin-B-like peptides from other Ranidae amphibians and to confirm this hypothesis.

Acknowledgments This work was supported by Chinese National Natural Science Foundation (30830021, 30800185, 31025025), the Ministry of Science and Technology (2010CB529800, 2009ZX09103-1/091) and the Ministry of Agriculture (2009ZX08009-159B).

Conflict of interest None.

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