Toxicon 55 (2010) 430–435

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Evidence from the primary structures of dermal antimicrobial peptides that *Rana tagoi okiensis* and *Rana tagoi tagoi* (Ranidae) are not conspecific subspecies

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ARTICLE INFO

Article history:
Received 19 June 2009
Received in revised form
17 September 2009
Accepted 18 September 2009
Available online 30 September 2009

Keywords:
Antimicrobial peptide
Brevinin-1
Ranatuerin-2
Temporin
Frog taxonomy

ABSTRACT

Morphological evidence and data from comparisons of nucleotide sequences of mitochondrial genes demonstrate considerable intraspecies variation among populations of the Japanese brown frog *Rana tagoi* Okada 1928 (Tago's brown frog). Five peptides with antimicrobial activity were isolated from an extract of the skins of specimens of *Rana tagoi okiensis* collected on the Oki Islands, Japan. Determination of their primary structures demonstrated that two peptides belong to the ranatuerin-2 family, two peptides to the temporin family, and one peptide to the brevinin-1 family. Ranatuerin-2 peptides were not previously identified in the skin of specimens of *R. t. tagoi* collected in Chiba Prefecture, Japan and the structures of the temporin peptides from *R. t. okiensis* (temporin-TOa: FLPILGKLLSGFL.NH_2 and temporin-TOb: FLPILGKLLSGLL.NH_2) are different from temporin-TGa (FLPILGKLLSGIL.NH_2) isolated from *R. t. tagoi*. Similarly, the acyclic C-terminally α-amidated brevinin-1 peptide from *R. t. okiensis* (Brevinin-1TOa, GIGSILGVIAKGLPTLIS-WIKNR.NH_2) shows three amino acid substitutions (Gly^1 → Ala, Val^8 → Ala, Ile^9 → Leu) compared to the ortholog from *R. t. tagoi*. In addition, bradykinin, identical to the mammalian peptide, is present in high concentration in the skin of *R. t. okiensis* but not *R. t. tagoi*. The data provide evidence to support the proposal that *R. t. tagoi* and *R. t. okiensis* should be regarded as separate species (*R. tagoi* and *R. okiensis*) rather than conspecific subspecies.

1. Introduction

The Japanese brown frogs are currently considered to comprise eight species (*Rana dybowskii*, *R. japonica*, *R. okinavana*, *R. ornativentris*, *R. pirica*, *R. sakuraii*, *R. tagoi*, and *R. tsushimensis*) (Maeda and Matsui, 1993). However, the taxonomy and evolutionary history of the group is complex (reviewed in Tanaka-Ueno et al., 1998) and several issues remain to be resolved. Because morphological differences are slight, some species (*R. dybowskii*, *R. ornativentris* and *R. pirica*) were considered to be conspecific with the European common frog *R. temporaria* until it was shown that the Japanese brown frogs and *R. temporaria* produced only sterile hybrids (Kawamura, 1962). More recently, the taxonomic status of "*Rana okinavana*" on the middle islands of the Ryukyu Archipelago has been called into question as the species may be identical to *R. psaltes* (now reclassified as *Babina okinavana*) (Matsui, 2007).

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0041-0101/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2009.09.010

The Japanese brown frogs may be divided into two classes on the basis of chromosome number. *R. japonica*, *R. okinavana, R. sakuraii, R. tagoi*, and *R. tsushimensis* have diploid chromosomes of $2n = 26$ whereas *R. dybowskii*, *R. ornativentris*, and *R. pirica* have $2n = 24$ (Green and Borkin, 1993). However, karyotypes of *R. tagoi* with chromosome number $2n = 28$ are found in the Chausu mountains of the Minamishinshu district of Nagano Prefecture (Ryuzaki et al., 2006).

The taxonomic status of the Japanese brown frogs is further complicated by demonstration of appreciable molecular heterogeneity within different populations of a particular species. Evidence for this statement is based upon comparisons of nucleotide sequences of mitochondrial genes (Tanaka et al., 1994, 1996; Sumida and Ogata, 1998; Sumida et al., 2003) and allozyme variations (Green and Borkin, 1993; Sumida and Nishioka, 1996; Trakimas et al., 2003). Intraspecies variation is greatest among the *R. tagoi* population and three sub-species have been proposed: *R. t. tagoi*, *R. t. yakushimensis*, and *R. t. okiensis* (Maeda and Matsui, 1993). Tago's brown frog *R. t. tagoi* is widely distributed in mountain regions of Honshu, Shikoku, and Kyushu islands whereas Oki Tago's brown frog *R. t. okiensis* and Yakushima Tago's brown frog *R. t. yakushimensis* are found only on the Oki and Yakushima islands, respectively. On the basis of comparison of nucleotide sequences of mitochondrial cytochrome *b* genes, it was proposed that *R. t. tagoi* and *R. t. yakushimensis* are sister-groups and more closely related to *R. sakuraii* than to *R. t. okiensis* (Tanaka et al., 1996). In contrast, analysis based upon the electrophoretic mobilities of enzymes and blood proteins suggested a closer relationship of *R. t. tagoi* with *R. t. okiensis* than with *R. t. yakushimensis* (Nishioka et al., 1987).

Peptide toxins with broad-spectrum antibacterial and antifungal activities and with the ability to lyse mammalian cells are synthesized in the skins of the majority of species of frogs belonging to the family Ranidae that have been studied to-date (Conlon, 2008; Conlon et al., 2009). These peptides probably represent a component of the system of innate immunity that defends the animal against invasion by pathogenic microorganisms (Hancock, 2001). The structures and biological activities of the antimicrobial peptides present in the skin extracts and/or skin secretions of the Japanese brown frogs have been studied in some detail (Conlon et al., 2003, 2004, 2005, 2006a, 2007b; Isaacson et al., 2002; Kim et al., 2001; Suzuki et al., 2007a). The nucleotide sequences of cDNAs encoding the biosynthetic precursors of several of the peptides have also been determined (Iwamuro et al., 2006; Koyama and Iwamuro, 2008; Ohnuma et al., 2006, 2007; Suzuki et al., 2007a, 2007b).

Previous studies have demonstrated that the amino acid sequences of antimicrobial peptides have value in inferring evolutionary relationships among frogs of the Ranidae family (Conlon et al., 2009) and the sequences of brevinin-2 peptides have been used to investigate the phylogenetic relationships among the Japanese brown frogs (Conlon et al., 2007b). The aim of the present study was to characterize the antimicrobial peptides present in an extract of the skin of *R. t. okiensis* and compare their primary structures and antimicrobial activities with those previously determined for peptides isolated from *R. t. tagoi* (Conlon et al., 2003). Nomenclature adopted for antimicrobial peptides from frogs of the Ranidae family follows recent guidelines (Conlon, 2008) and species nomenclature follows the taxonomic recommendations of Frost (2009).

2. Experimental

2.1. Tissue collection and extraction

All experiments were approved by Toho University Bioethics and Animal Ethics Committee and were carried out by authorized investigators. Adult and sub-adult specimens of *R. t. okiensis* ($n = 31$, three females; body weight range 3.6–10.6 g; length 34–54 mm) were collected in February at two sites in the Oki Islands, Shimane Prefecture, Japan. The animals were anesthetized by immersion in ice-water and sacrificed by decapitation. Skin was immediately removed and freeze-dried for shipment to U.A.E. University. The tissue (2.1 g) was extracted by homogenization in ethanol/0.7 M HCl (3:1 v/v; 100 ml) at 0 °C using a Waring blender. The homogenate was stirred for 1 h at 0 °C and centrifuged (4000 × *g* for 30 min at 4 °C). Ethanol was removed from the supernatant under reduced pressure and, after further centrifugation (4000 × *g* for 30 min at 4 °C), the extract was pumped onto 8 Sep–Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series at a flow rate of 2 ml/min. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0:29.9:0.1, v/v/v) and freeze-dried.

For the purposes of comparison, specimens ($n = 5$, two females; body weight range 3.0–5.4 g; length 34–43 mm) of *R. t. tagoi* were collected in Ichihara City, Chiba Prefecture, Japan in March. The tissue (2.7 g) was extracted and processed in the same way as the *R. t. okiensis* tissue.

2.2. Antimicrobial assays

Purification of the peptides was monitored by incubating lyophilized aliquots of chromatographic effluent in Mueller–Hinton broth (50 μl) with an inoculum (50 μl of 10^6 colony forming units/ml) from a log-phase culture of reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) in 96-well microtiter cell-culture plates for 18 h at 37 °C in a humidified atmosphere of air. After incubation, the absorbance at 630 nm of each well was determined using a microtiter plate reader. Minimum inhibitory concentration (MIC) against *S. aureus*, *E. coli*, and *Candida albicans* (ATCC 90028) was measured by standard microdilution methods (Clinical and Laboratory Standards Institute, 2008a,b). Incubations with *C. albicans* were carried out in RPMI 1640 medium for 48 h at 35 °C. In order to monitor the validity and reproducibility of the assays, incubations were carried out in parallel with increasing concentrations of ampicillin for reference strains of bacteria and amphotericin for *C. albicans* as previously described (Conlon et al., 2007b).

2.3. Peptide purification

The skin extract, after partial purification on Sep–Pak cartridges, was redissolved in 0.1% (v/v) trifluoroacetic