

Unmasking venom gland transcriptomes in reptile venoms

Tianbao Chen,^{a,b} Anthony J. Bjourson,^a David F. Orr,^a HangFai Kwok,^a Pingfan Rao,^b
Craig Ivanyi,^c and Chris Shaw^{a,*}

^a *Pharmaceutical Biotechnology, School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, Ireland*

^b *Institute of Biotechnology, University of Fuzhou, Fuzhou, Fujian 350002, PR China*

^c *Arizona-Sonora Desert Museum, Kinney Road, Tucson, AZ 85743-8918, USA*

Received 13 June 2002

Abstract

While structural studies of reptile venom toxins can be achieved using lyophilized venom samples, until now the cloning of precursor cDNAs required sacrifice of the specimen for dissection of the venom glands. Here we describe a simple and rapid technique that unmask venom protein mRNAs present in lyophilized venom samples. To illustrate the technique we have RT-PCR-amplified a range of venom protein transcripts from cDNA libraries derived from the venoms of a hemotoxic snake, the Chinese copperhead (*Deinagkistrodon acutus*), a neurotoxic snake, the black mamba (*Dendroaspis polylepis*), and a venomous lizard, the Gila monster (*Heloderma suspectum*). These include a metalloproteinase and phospholipase A2 from *D. acutus*, a potassium channel blocker, dendrotoxin K, from *D. polylepis*, and exendin-4 from *H. suspectum*. These findings imply that the apparent absence and/or lability of mRNA in complex biological matrices is not always real and paves the way for accelerated acquisition of molecular genetic data on venom toxins for scientific and potential therapeutic purposes without sacrifice of endangered herpetofauna.

© 2002 Elsevier Science (USA). All rights reserved.

Snake bite is an ever present threat for many communities in both developed and underdeveloped nations [1–4]. Although antivenins have gone a long way to reduce mortality, their production remains a hit-or-miss affair with many not achieving optimal protective effects [5]. For this reason, the understanding of venom component toxicology resulting from biochemical, pharmacological, and molecular biological studies remains paramount in the quest for optimized therapeutic reagents. Acquisition of venom samples from snakes for biochemical and pharmacological studies, while hazardous to the human handler, does not unduly harm the specimen and many snakes are farmed for such purposes with no observable long-term deleterious effects [6,7]. However, acquiring molecular genetic information on precursor structures derived from cloned cDNAs requires sacrifice of the specimens with subsequent venom gland dissection [8–11].

In addition to the production of antivenins, snake venom research has a high potential for novel thera-

peutic drug discovery, reflecting perhaps a reawakening of forgotten wisdom from ancient cultures [12]. Many venom proteins do indeed possess powerful therapeutic properties including fibrinolysis [13], inhibition of platelet aggregation [14], induction of apoptosis of endothelial cells [15], and conversely, induction of vascular endothelial cell growth [9]. In fact, one of the top-selling drug classes in Western medicine for the treatment of hypertension, the ACE-inhibitors, evolved from structure/activity studies on the bradykinin-potentiating peptides from Brazilian lance-headed viper (*Bothrops jararaca*) venom [16].

Many species of reptile are endangered or threatened for a variety of reasons, such as habitat destruction or human practices involving either traditional mass culls or culinary purposes. The emerging science of functional genomics integrates data derived from proteomics, genomics, and physiomics to provide a holistic view of the life process—a necessary prerequisite for a global understanding. In the case of snake venom proteomics, where many toxic proteins are of relatively high molecular mass, direct sequencing to obtain full primary structure is not the method of choice. Most

* Corresponding author. Fax: +44-2870-324965.
E-mail address: c.shaw@ulster.ac.uk (C. Shaw).

researchers rather resort to molecular cloning to this end using either degenerate primers designed for small sequence tags derived from the protein of interest or those designed for conserved nucleic acid domains of related molecules from other species. Here we describe a simple and rapid technique for the cloning of venom gland protein cDNAs from lyophilized venom samples that does not require sacrifice and dissection of the specimen.

Materials and methods

Biological materials

The venoms from three species of venomous reptile were chosen for illustration purposes and each was secured via very different routes and sampling conditions, perceived as being suboptimal in the extreme for recovery of labile mRNA. The Chinese copperhead (*Deinagkistrodon acutus*), a crotalid snake, has a hemotoxic venom and is responsible for many snake bite mortalities in southern China. Venom was obtained from snakes farmed in the Wuyishan region of Fujian Province and underwent a 300-mile rail journey at ambient temperature before lyophilization. Lyophilized venom (10 mg) from the black mamba (*Dendroaspis polylepis*), an African elapid snake with a potent neurotoxic venom, was obtained from the Sigma Chemical Company. This lot of venom (118F0275) was prepared for Sigma in 1988 and had been stored below 0°C prior to dispatch to our laboratory in 2002. Venom from the Gila monster (*Heloderma suspectum*), one of only two species of venomous lizards from south western United States and Mexico, was obtained from the Arizona-Sonora Desert Museum in Tucson, Arizona. Gila monster envenomation produces a rapid and profound lowering of blood pressure. As this species is a Convention in International Trade in Endangered Species (CITES) Appendix II designate, all necessary permits for export and import of venom were obtained from both the United States Department of Agriculture and the British Customs and Excise, in accordance with the international treaty.

Sample preparation

Lyophilized reptile venoms (10 mg) were reconstituted in 1 ml of lysis buffer consisting of 500 mM LiCl, 10 mM EDTA, 1% (w/v) SDS, and 5 mM dithiothreitol in 100 mM Tris-HCl, pH 8.0. Polyadenylated (poly(A)) mRNA was isolated from reconstituted venoms by incubation with 250 µl of prewashed, magnetized oligo(dT) beads for 10 min, followed by several sequential washes of the beads as described by the manufacturer (Dyna, UK). mRNA was eluted from the beads using

20 µl of 10 mM Tris-HCl, pH 8.0, at 75 °C for 2 min and reverse-transcribed using oligo (dT)-CDS primer and Powerscript reverse transcriptase (Clontech, UK) at 42 °C for 90 min. For each venom, a duplicate control reaction, in which reverse transcriptase was omitted, was also performed to exclude the possibility of results being obtained from contaminating DNA. Reaction mixtures were diluted to 60 µl with sterile, deionized water and 6 µl of each dilution was PCR-amplified for 35 cycles (each 94 °C for 30 s; 66 °C for 30 s; 72 °C for 3 min) using thermostable polymerase (Invitrogen) and appropriate sense and antisense primers for each venom protein cDNA. The primers used were designed using the VECTOR NTI package, and the primer sequences, regions amplified, and expected product sizes are as shown in Table 1. Ten-microliter samples from each reaction were subjected to gel electrophoresis and photographed using standard procedures. PCR products were sequenced directly using a dye terminator cycle sequencing kit and an ABI 3100 automated DNA sequencer (Applied Biosystems).

Results and discussion

To effect isolation of mRNA from snake venom gland tissues, the most commonly employed technique involves total RNA precipitation using organic solvent-based extraction media [8–11,13–15,17–19]. The subpopulations of mRNAs of interest are coprecipitated with other RNA types that are predominant. The origin of this mRNA is assumed to be the cytosolic compartment. Using this isolation approach on lyophilized venom samples fails to recover appropriate RT-PCR-compatible mRNA and hence its absence was assumed. However, the employment of magnetized oligo-dT DynaBeads for polyadenylated mRNA capture and circumvention of precipitation recovers venom protein poly(A) mRNA which is RT-PCR-compatible and representative of all the studied classes of protein from reptile venoms presented here. Metalloproteinase [17] and phospholipase A2 [18] were targeted from *D. acutus* venom, dendrotoxin K [19], a potassium channel blocker, was targeted from *D. polylepis* venom; exendin-4 [20], a glucagon-like peptide 1 receptor-agonist, was targeted from *H. suspectum* venom. All nucleotide sequences of cloned cDNAs were obtained from the EMBL database and both sense and antisense primers for RT-PCR were designed from appropriate regions (Table 1). Following first-strand and second-strand cDNA synthesis from the captured poly(A) mRNA, RT-PCR was performed with each set of primers and the results are shown in Fig. 1. All of the single PCR products generated were of the expected size, and direct nucleotide sequencing, followed by trawling of unedited data on the EMBL database, confirmed unequivocally

Table 1
Details of venom protein precursor cDNAs targeted and primers employed for RT-PCR amplification

| Species | Venom protein and Accession No. | Sense primer | Antisense primer | Region amplified (bp) | Product size (bp) |
|---------------------|---------------------------------|----------------------------|----------------------------|-----------------------|-------------------|
| <i>D. acutus</i> | Metalloproteinase - AJ223283 | GCCCATCAAAAAGGCCTCTCAGTTA | TGCTCGTTAGGCATGGTAGGGATTT | 549–1464 | 916 |
| <i>D. acutus</i> | Phospholipase A2 - AJ223188 | GCTCTTTGGATAGTGGCCGTGTTG | GCAGATTGCGAAGGCCTTGTC | 7–333 | 327 |
| <i>D. polylepis</i> | Dendrotoxin K - S61886 | ACCCCTGTCTCTGGCCGTGCAAAAGT | CAGAGAAAAAGGAATGAGCCACAGGG | 53–365 | 312 |
| <i>H. suspectum</i> | Exendin 4 - U77613 | TGGCTGTGTGTTTTGGGCTGTCC | GGCTGTGTCGCTCACTAATTGGAAA | 79–439 | 361 |

the relevant identity of each product (Table 2). PCR of libraries generated in the absence of reverse transcriptase failed to generate products precluding somatic DNA in the venom as a source of template (Fig. 1).

Polyadenylated mRNAs, constituting representative products of the venom gland transcriptome of hemotoxic and neurotoxic venomous snakes and a venomous lizard, are present in secreted venom despite procurement or storage of original samples under conditions that would favor rapid degradation. Conventional RNA isolation protocols used prior to this discovery have failed to recover mRNA in a RT-PCR-compatible form, leading to the assumptions that either transcriptome message is not present or it has been degraded. Venoms from many invertebrates and vertebrates are rich in amphipathic/cationic peptides that have been shown to possess nucleic acid binding properties and indeed to afford protection against degradation in gene transfection experiments [21,22]. While the nature of the molecule(s) affecting protection of labile mRNAs in reptile venom remain to be elucidated, unpublished experiments in our laboratory have shown that cDNAs incubated with synthetic amphipathic/cationic peptides are coprecipitated as complexes using standard nucleic acid precipitation protocols with organic solvent. However, solid-phase partitioning of such complexes, followed by an elevation in pH with several buffer washes, effectively reverses this interaction. Incubation of *D. acutus* metalloproteinase RT-PCR product with 100 µg of *D. acutus* venom for 30 min at 25 °C resulted in a significant retardation following gel electrophoresis, indicating interaction with an endogenous nucleic acid binding component in the venom [23,24] (Fig. 2). In our laboratory, we have found that incubation of RT-PCR products with synthetic amphipathic peptides produces the same gel retardation effect as demonstrated here with crude venom. The time course of the interaction of nucleic acid with both crude venom and amphipathic peptides is of the order of 3–15 min, dependent upon source of material (unpublished results). For this reason, 30-min incubations were employed as routine to ensure that the interactions had gone to completion. We conclude that nucleic acid (mRNA) protection in reptile venom is afforded by such as yet unidentified components and that the procedure employed in this study effectively unmask the transcriptome of the venom gland.

The simple protocol described here should circumvent the necessity of sacrifice of many snakes hitherto killed for their venom glands in the pursuit of what are essentially molecular biological approaches to the structural characterization of toxic venom proteins. In addition, the way has been paved to facilitate an accelerated acquisition of this information for modern biotechnological applications. Urgent advances are required in recombinant venom protein production and the development of DNA vaccines for antivenin gen-

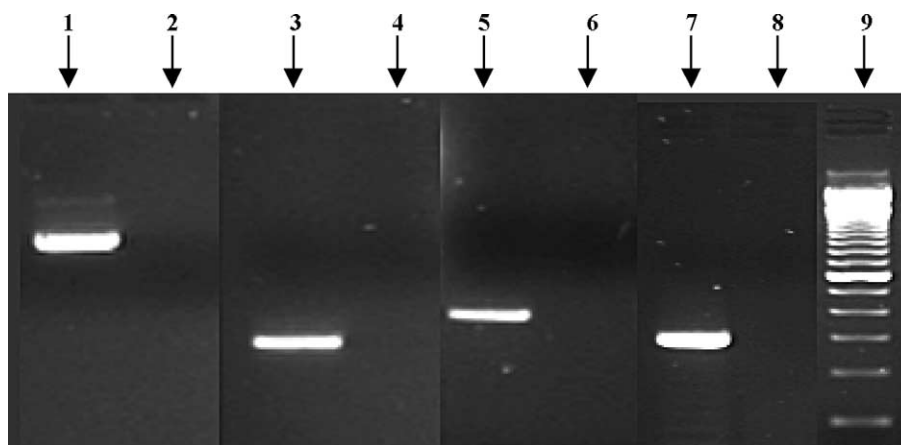


Fig. 1. RT-PCR amplification of mRNA encoding protein transcripts from lyophilized reptile venoms. RT-PCR products from cDNA libraries of *D. acutus* (Chinese copperhead) lyophilized venom using metalloproteinase (aculysin-2) primers (lane 1) and phospholipase A2 primers (lane 3), *H. suspectum* (Gila monster) venom using primers to extendin-4 precursor (lane 5), and *D. polylepis* (black mamba) venom using dendrotoxin K1 primers (lane 7). Lanes 2, 4, 6, and 8 represent control reactions where reverse transcriptase was omitted from each reaction. Lane 9 contains a standard DNA ladder, each band representing 100 bp increments.

Table 2

Identity of unedited direct nucleotide sequences from reptile venom RT-PCR products using WU-BLAST 2

| No. | Result |
|-----|--|
| 1 | AJ223283 <i>Agkistrodon acutus</i> mRNA for aculysin-2 Length = 1476 Plus Strand HSPs: Score = 4210 (637.7 bits), Expect = 9.7e-184, P = 9.7e-184 Identities = 842/842 (100%) , Positives = 842/842 (100%) , Strand = Plus/Plus |
| 2 | AJ223188 <i>Agkistrodon acutus</i> Mrna for phospholipase A2 Length = 429 Plus Strand HSPs: Score = 1635 (251.4 bits), Expect = 6.7e-67, P = 6.7e-67 Identities = 327/327 (100%) , Positives = 327/327 (100%) , Strand = Plus/Plus |
| 3 | U77613 <i>Heloderma suspectum</i> extendin 4 mRNA, complete cds. Length = 465 Plus Strand HSPs: Score = 1805 (276.9 bits), Expect = 1.3e-74, P = 1.3e-74 Identities = 361/361 (100%) , Positives = 361/361 (100%) , Strand = Plus/Plus |
| 4 | S61886 dendrotoxin K = K ⁺ channel blocker [<i>Dendroaspis polylepis</i> = black mamba, mRNA Partial, 378 nt]. Length = 378 Plus Strand HSPs: Score = 1509 (232.5 bits), Expect = 3.7e-61, P = 3.7e-61 Identities = 309/315 (98%) , Positives = 309/315 (98%) , Strand = Plus/Plus |

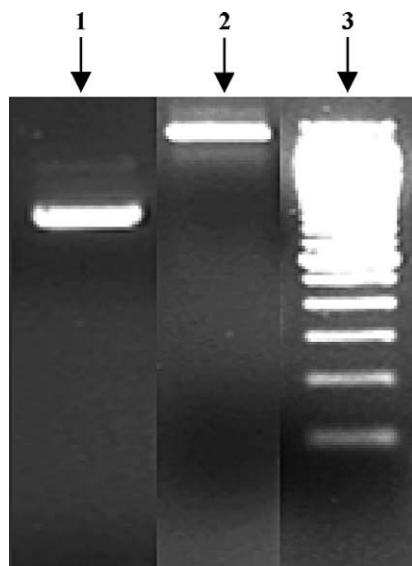


Fig. 2. Gel retardation experiment electropherogram. A 916-bp RT-PCR product (lane 1) from the *D. acutus* aculysin-2 precursor mRNA was incubated for 30 min at 25 °C with 100 µg of *D. acutus* lyophilized venom reconstituted in 5 µl of distilled-deionized water (lane 2). The presence of venom clearly retarded the DNA fragment, indicating a molecular interaction with endogenous venom component(s).

eration to meet what is becoming a critical need for snake bite therapeutics, especially in the developing world [25–27]. While the snake has from ancient times symbolized both healing and death [12], perhaps this timely report can in some part aid in tipping the contemporary scales toward the fuller realization of the former, while positively addressing aspects of global biodiversity conservation and ethical use of animals in research.

Acknowledgments

Tianbao Chen and HangFai Kwok were both in receipt of Vice Chancellor's Research Studentships from the University of Ulster. We thank the staff of the Sonoran-Arizona Desert Museum for acquisition of venom from Gila monsters (*H. suspectum*) and for their efforts in securing necessary documentation under CITES and USDA export regulations. We also are grateful to the Fujian Provincial Science and Technology Department, China for a research project grant (2000Z114).

References

- [1] R.C. Dart, J. McNally, Efficacy safety and use of snake antivenoms in the United States, *Ann. Emer. Med.* 37 (2001) 181–188.
- [2] Q.B. Li et al., Hemostatic disturbances observed in patients with snakebite in south China, *Toxicon* 38 (2000) 1355–1366.
- [3] N. Al Harbi, Epidemiological and clinical differences in snake bites among children and adults in south western Saudi Arabia, *J. Acc. Emer. Med.* 16 (1999) 428–430.
- [4] M. Miller, Amateur reptile keepers and antivenom consumption, *Emer. Med.* 13 (2001) 126–127.
- [5] S.W. Nkinin et al., Genetic origin of venom variability: impact on the preparation of antivenin serums, *Bull. Soc. Path. Exotique* 90 (1990) 277–281.
- [6] S.A. Nishioka, P.V. Silveira, F.M. Peixoto-Filho, M.T. Jorge, A. Sandoz, Occupational injuries with captive lance-headed vipers (*Bothrops moojeni*): experience from a snake farm in Brazil, *Trop. Med. Int. Health* 5 (2000) 507–510.
- [7] L. Chanhom, P. Jintakune, H. Wilde, M.J. Cox, Venomous snake husbandry in Thailand, *Wilderness Environ. Med.* 12 (2001) 17–23.
- [8] J. Silveira de Oliveira et al., Cloning and characterization of an alpha-neurotoxin-type protein specific for the coral snake *Micrurus corallinus*, *Biochem. Biophys. Res. Commun.* 267 (2000) 887–891.
- [9] I.L. Junqueira de Azevedo, F.H. Farsky, S.H. Oliveira, P.L. Ho, Molecular cloning and expression of a functional snake venom vascular endothelium growth factor (VEGF) from the *Bothrops insularis* pit viper. A new member of the VEGF family of proteins, *J. Biol. Chem.* 276 (2001) 39836–39842.
- [10] H.G. Zha, W.H. Lee, Y. Zhang, Cloning of cDNAs encoding C-type lectins from Elapidae snakes *Bungarus fasciatus* and *Bungarus multicinctus*, *Toxicon* 39 (2001) 1887–1892.
- [11] Y.W. Guo et al., Cloning and functional expression of mucrosobin protein, a beta-fibrinogenase of *Trimeresurus mucrosquamatus* (Taiwan Habu), *Prot. Exp. Purif.* 23 (2001) 483–490.
- [12] I.A. Ramoutsaki, S. Haniotakis, A.M. Tsatsakis, The snake as the symbol of medicine, toxicology and toxinology, *Vet. Human Toxicol.* 42 (2000) 306–308.
- [13] X. Cheng et al., Purification, characterization, and cDNA cloning of a new fibrinogenolytic venom protein, Agkisacutacin, from *Agkistrodon acutus* venom, *Biochem. Biophys. Res. Commun.* 265 (1999) 530–535.
- [14] Y.S. Koh, D.S. Kim, Characterization and cDNA cloning of a platelet aggregation inhibitor, *Mol. Cell.* 10 (2000) 437–442.
- [15] W.B. Wu, S.C. Chang, M.Y. Liao, T.F. Huang, Purification, molecular cloning and mechanism of action of graminelysin I, a snake venom-derived metalloproteinase that induces apoptosis of human endothelial cells, *Biochem. J.* 357 (2001) 719–728.
- [16] B.J. Hawgood, Mauricio Rocha e Silva MD: snake venom, bradykinin and the rise of autopharmacology, *Toxicon* 35 (1997) 1569–1580.
- [17] I.H. Tsai, Y.M. Wang, T.Y. Chiang, Y.L. Chen, R.J. Huang, Purification, cloning and sequence analyses for pro-metalloprotease-disintegrin variants from *Deinagkistrodon acutus* venom and subclassification of the small venom metalloproteases, *Eur. J. Biochem.* 267 (2000) 1359–1367.
- [18] C.Y. Fan, Y.C. Qian, S.L. Yang, Y. Gong, cDNA cloning and sequence analysis of Lys-49 phospholipase A2 from *Agkistrodon acutus*, *Gen. Anal. Biomol. Eng.* 15 (1999) 15–18.
- [19] L.A. Smith, P.J. Lafaye, H.F. LaPenotiere, T. Spain, J.O. Dolly, Cloning and functional expression of dendrotoxin K from black mamba, a K⁺ channel blocker, *Biochemistry* 32 (1993) 5692–5697.
- [20] M. Pohl, S. Wank, A Molecular cloning of the helodermin and exendin-4 cDNAs in the lizard. Relationship to vasoactive intestinal polypeptide/pituitary adenylate cyclase activating polypeptide and glucagon-like peptide 1 and evidence against the existence of mammalian homologues, *J. Biol. Chem.* 273 (1998) 9778–9784.
- [21] M. Wachinger et al., Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression, *J. Gen. Virol.* 79 (1998) 731–740.
- [22] T.B. Wyman et al., Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers, *Biochemistry* 36 (1997) 3008–3017.
- [23] J. Yamane, K. Makino, T. Morii, Y. Sugiura, DNA recognition by peptide oligomers, *Nucleic Acids Symp. Series.* 34 (1995) 143–144.
- [24] M.R. Alam, M. Maeda, S. Sasaki, DNA-binding peptides searched from the solid-phase combinatorial library with the use of the magnetic beads attaching the target duplex DNA, *Bioorg. Med. Chem.* 8 (2000) 465–473.
- [25] R.A. Borgheresi, S. Palma, F. Duncancel, A.C. Camargo, E. Carmona, Expression and processing of recombinant sarafotoxins precursor in *Pichia pastoris*, *Toxicon* 39 (2001) 1211–1218.
- [26] R.A. Harrison, A. Richards, G.D. Laing, R.D.G. Theakston, Simultaneous GeneGun immunisation with plasmids encoding antigen and GM-CSF: significant enhancement of murine anti-venom IgG1 titres, *Vaccine* 20 (2002) 1702–1706.
- [27] R.D. Theakston, D. Warrell, A Crisis in snake antivenom supply for Africa, *Lancet* 356 (2000) 2104.