Toxicon 186 (2020) 105–108

Contents lists available at ScienceDirect

Toxicon

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

Asymmetrical expression of toxins between the left and right venom glands of an individual prairie rattlesnake (*Crotalus viridis viridis*)

Cara Francesca Smith, Stephen P. Mackessy *

School of Biological Sciences, University of Northern Colorado, 501 20th Street, Greeley, CO, 80639-0017, USA

A B S T R A C T

It is assumed that toxin expression is equivalent between left and right glands of a single snake. In the current study, we report venoms that differ in enzyme functionality and overall composition between the left and right gland of a single snake. The right gland produced venom of comparable composition to venom previously extracted from the same individual; however, the left gland produced venom with overall lower protein content and considerably less enzyme activity.

Snake venoms are complex toxic mixtures composed primarily of potent bioactive proteins used for prey incapacitation or defense. Venom compositional variation is a ubiquitous phenomenon that has been observed in a variety of contexts throughout venomous snake taxa. Previously, venom composition has been shown to vary taxonomically, ontogenetically, geographically, and based on dietary preference (Mackessy, 1988, 2010; Chippaux et al., 1991; Mackessy et al., 2003; Massey et al., 2012). Understanding these types of venom variation has significant evolutionary implications and can inform snakebite treatment (Massey et al., 2012).

There are recorded cases of individual snakes within a species expressing aberrant white venoms that differed in activity of major enzymes and overall electrophoretic profiles compared to 'normal' yellow venoms (Kornalik and Master, 1964; Dimitrov and Kankornkar, 1968). However, it is typically assumed that within a single snake, venom toxin expression is equivalent between left and right glands. This has been previously assumed in a variety of studies that either pool venom from both glands (e.g. Smith and Mackessy, 2016; Mackessy, 1988; 2003; 2010; Chippaux et al., 1982) or assume comparable transcript expression between left and right glands (Aird et al., 2015; Rokyta et al., 2012).

While this assumption has been generalized to venom expression in snakes, there has been a single recorded instance of unequal toxin expression between glands in an individual *Crotalus oreganus helleri* (Johnson et al., 1987). The white and yellow venoms of this individual displayed differences in toxicity, intradermal hemorrhagic activity, enzyme activity and overall protein content, and venoms from both glands produced myonecrosis in mouse muscle. Here we report expression in a seemingly normal right gland of this species and compare it to aberrant white venom produced in the left gland and to a previously extracted and pooled sample. Though this phenomenon has been observed once previously, to our knowledge this is the first evidence of aberrant expression in a previously normal gland.

A single *Crotalus v. viridis* rattlesnake was collected from Weld County, Colorado in 2017 (Colorado Parks and Wildlife permit #14HP974). The snake was processed in the UNC Animal Resource Facility and returned to the location of capture (IACUC protocol # 1302D-SM-S-16). The snake was scanned for a PIT (passive integrated transponder) tag (AVID, Norco, CA) and was found to be a previously captured individual with a single capture date in 2014. The snake was an adult female, with snout to vent/tail lengths of 640/37 (2014) and 715/41 (2017); therefore, ontogenetic effects (Saviola et al., 2015) were not a concern. Venom was manually extracted as previously described (Mackessy, 1988); however, left and right gland capillary tube volumes were not pooled as usual, because of their differing appearance upon extraction. While the physiological cause of the gland abnormality was unknown, differences in venom quality between the left and right glands was initially evaluated based on thousands of previous extractions of this species. Both samples were frozen at −80 °C, lyophilized and stored at −20 °C. For all assays and analyses, left and right gland venoms collected in 2017 were compared to venom collected and pooled from the left and right glands from the same snake in 2014, which was apparently normal at extraction.

Extracted crude venom protein concentration was estimated using dry weight of venom and venom volume measured at extraction. Dried left and right gland venom samples (and 2014 sample) were reconstituted at an apparent concentration of 4.0 mg/mL in Millipore-filtered water, vortexed, centrifuged for 5 min at 9500×*g*. Protein concentration of the supernatant was determined using the Pierce™ BCA Protein Assay

* Corresponding author.
E-mail address: stephen.mackessy@unco.edu (S.P. Mackessy).

https://doi.org/10.1016/j.toxicon.2020.08.005
Received 6 July 2020; Received in revised form 1 August 2020; Accepted 5 August 2020
Available online 7 August 2020

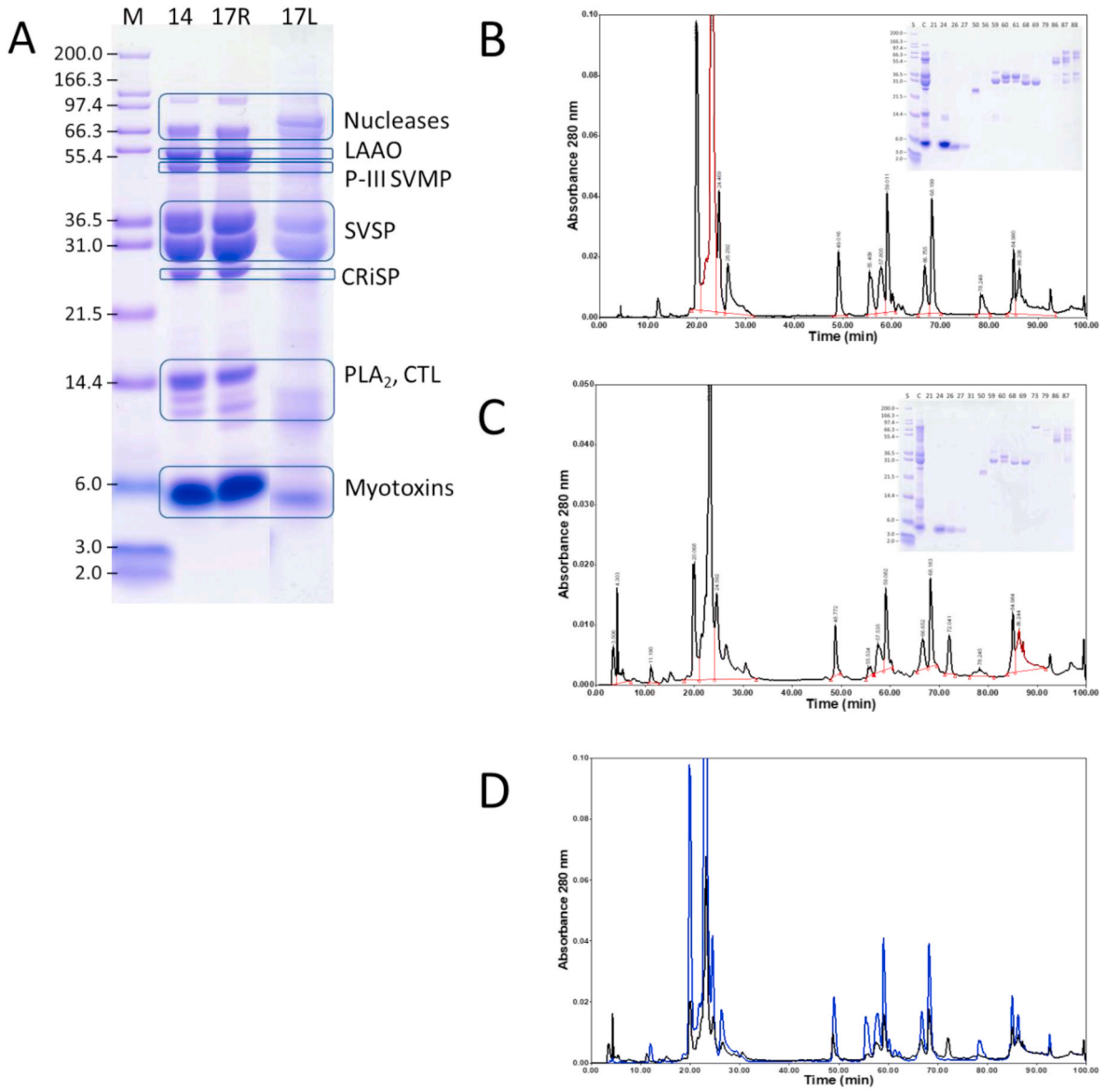

Fig. 1. SDS-PAGE of 20 μg crude venom (reduced) from 2014 (14), 2017 right gland (17R) and 2017 left gland (17L) and protein size standards (M). Toxin families are indicated on the right based on Saviola et al. (2015) and Mackessy (2010) (A). HPLC chromatograms and protein gels of separated fractions (reduced) of 0.8 mg of *C. v. viridis* crude venom from right gland extracted in 2017 (B), from left gland extracted in 2017 (C), and overlay of right (blue) and left (black) gland chromatograms to show detail (D). The same amount of protein (based on protein determination assays) was added to the crude venom gel and for HPLC analysis. Note that in general, peaks correspond between samples, but there is a considerably lower amount of protein from the left gland. CRiSP, cysteine-rich secretory protein; CTL, C-type lectins; LAAO, L-amino acid oxidase; PLA_2, phospholipase A_2; P-III SVMP, P-III snake venom metalloproteinase; SVSP, snake venom serine proteinase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Kit protocol with bovine γ globulin as a standard (Rockford, IL), and the amount of material used in all subsequent assays was based on these determinations. Reconstituted samples were frozen at −20 °C until use and then thawed and centrifuged at 9500×*g* for 5 min to pellet cellular debris.

Crude venom (20 μg) or lyophilized protein (approximately 5 μg - RP-HPLC fractionated) was reduced with DTT and loaded into wells of a NuPAGE Novex Bis-Tris 12% acrylamide Mini Gel as previously described (Smith and Mackessy, 2016). Densitometry analysis was performed with ImageJ software (version 1.51j8), and relative densities of band clusters of known toxin families (Fig. 1A) were compared between samples by taking area under the curve of peaks representing band size and darkness. Azocasein metalloproteinase, L-amino acid oxidase, thrombin-like serine proteinase, and kallikrein-like serine proteinase assays were performed as outlined in Smith and Mackessy (2016). Phospholipase A_2 assays were performed as outlined in Reynolds et al. (1992). In brief, 0.5 μg of crude venom was combined with 10 μl of 10 mM DTNB (dithionitrobenzoic acid) in 0.4 M tris-HCl pH 8.0 and 200 μl of diheptanoyl thio-phosphatidyl choline in assay buffer (25 mM tris-HCL, pH 7.5 with 10 mM $CaCl_2$, 100 mM NaCl and 0.3 mM Triton X-100). The reaction absorbances were read at 37 °C every minute for 10 min at 414 nm, and specific activity was expressed as μmol product/min/μg venom protein (using the linear portion of the curve, 1–3 min).

Venom was subjected to reverse phase HPLC using a Waters system, Empower software and a Phenomenex Jupiter C_{18} (250 × 4.6 mm, 5 μm, 300 Å pore size) column as outlined in Smith and Mackessy (2016). Peptide/protein was detected at 220 nm and 280 nm with a Waters 2487 Dual λ Absorbance Detector. Fractions corresponding to each peak were then frozen at −80 °C overnight, lyophilized and then analyzed along

Table 1
Comparison of crude venom protein concentration and enzyme specific activities of venoms from a single individual extracted in 2014 (combined) and extracted in 2017 separately from the right and left gland. Note that the left gland displays almost complete loss of LAAO and PLA_2 activity in comparison to the right gland.

ID	Concentration (μg/μl)	LAAO	SVMP	Thr-SVSP	Kal-SVSP	PLA_2
2014–237	202.4	57.59	0.50	1192.35	368.33	9.04
2017–279R	304.7	96.84	0.53	1507.28	276.70	10.32
2017–279L	8.2	3.26	0.32	774.27	221.48	0.30
Percent Reduction	97.31	96.6	39.0	48.6	20.0	97.1

LAAO: L-amino acid oxidase (nmol product/min/mg protein). SVMP: Azocasein metalloproteinase (Abs342 nm/min/mg). Thr-SVSP: Thrombin-like serine proteinase (nmol product/min/mg). Kal-SVSP: Kallikrein-like serine proteinase (nmol product/min/mg). PLA_2: phospholipase A_2 (nmol product/min/mg). Percent reduction is comparison between R and L glands, 2017. Venom amounts for all assays were based on BCA protein assays.

with 20 μg crude venom via SDS-PAGE as described above in order to determine peak complexity, mass and toxin families.

During venom extraction, left gland material appeared turbid and white compared to venom expressed from the right fang, which was clear and bright yellow. Following centrifugation, a large pellet of cell debris was observed in the tube of the left gland, and the supernatant was removed and subsequently used for all analyses. Following lyophilization, dried venom from the right gland was the same color and consistency observed in other *C. viridis*, that is, dense powder and light yellow in color, whereas the lyophilized left gland venom was white and flaky.

Crotalus v. viridis venom in general is yellow, clear and viscous, with a high abundance of larger molecular weight enzymes and small, basic myotoxins (Saviola et al., 2015). While the overall ratios of major venom toxins were similar, total protein, enzyme activity and toxin levels were markedly reduced in the left gland. Protein concentration determination, HPLC, enzymology, SDS-PAGE and gel band densitometry analysis of crude venom (20 μg) revealed a marked decrease in the abundance of all toxin families of the left gland, though in general the same toxin families were present in right and left glands (Fig. 1A–D).

Venom from the right gland was equivalent to the normal sample extracted in 2014 and to the previously published venom proteome of *C. v. viridis* (Fig. 1A and B; Saviola et al., 2015). Observed protein concentration and common venom enzyme activities were noticeably reduced in the left gland venom compared to both the right gland and to pooled venom from the same individual from three years prior (Table 1). The left gland lost almost complete functionality of both PLA_2 and L-AAO activity, but thrombin-like SVSP, kallikrein-like SVSP, and SVMP activities were only reduced by 48.6%, 20% and 39%, respectively (Table 1). Therefore, it appears that certain toxin families are unequally affected in the white venom.

White venom had a similar gel banding pattern compared to yellow venom but contained more bands of high molecular weight proteins between ~50 and 70 kDa, and the PLA_2 band at ~14 kDa was virtually absent (Fig. 1A). At least 3 myotoxin isoforms are present in *C. v. viridis* venom (Saviola, 2015), but only a small amount of myotoxin was expressed in venom from the left gland. Though a concentration of 20 μg (BCA assay) of crude venom was loaded per lane, the left gland still appears to have a lower protein content overall. Densitometry analysis of crude venom gels revealed that left gland SVSPs, PLA_2, and myotoxin levels were 57%, 91% and 63% lower than in the right gland. Right gland densitometry for major band clusters matched the 2014 pooled venom.

Though it is typically assumed that venom produced in the left and right glands will be proteomically identical, this observation represents a case of inequivalent venom gland toxin production and expression. This case is particularly interesting because it demonstrates that a gland that produced 'normal' venom can change after reaching adulthood, and that certain toxin families may lose complete functionality while others are only partially reduced in activity. The unusual appearance and overall decreased toxin activity of the left gland venom may have been due to localized injury or infection of the venom gland, potentially causing misfolding of proteins or affecting the activity of pH-sensitive enzymes. It has recently been shown that venom gland physiology may be regionally heterogenous, with different cell populations producing different toxins (Post et al., 2020). If this regionalization is generally present in snake venom glands, then damage to a functionally compartmentalized venom gland could lead to an unequal effect on toxin expression.

The snake appeared healthy during extraction, so it is likely that prey capture in this individual would not have been affected due to the asymmetric functionality of the venom glands. However, it is unclear if this lack of symmetrical venom production and functionality would have long-term fitness consequences for this individual. It is also unclear if this change in toxin expression and venom phenotype is reversible. Though in general venom production should be equivalent between glands in a single snake, our results indicate that if venoms are visually different upon extraction, they should not be pooled, as glands may differ in toxin production and this could influence conclusions about overall venom composition and activity.

Ethical statement

The authors hereby state that all procedures involving animals were conducted in a humane and ethical manner. All protocols were evaluated and approved (prior to initiating research) by the University of Northern Colorado Institutional Animal Care and Use Committee (UNC-IACUC).

CRediT authorship contribution statement

Cara Francesca Smith: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Stephen P. Mackessy:** Conceptualization, Data curation, Writing - review & editing.

Acknowledgements

Support for this project was provided by a grant from the US National Science Foundation (DEB-1655571).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2020.08.005.

References

Aird, S.D., Aggarwal, S., Villar-Briones, A., Tin, M.M.Y., Terada, K., Mikheyev, A.S., 2015. Snake venoms are integrated systems, but abundant venom proteins evolve more rapidly. BMC Genom. 16, 647.

Chippaux, J.P., Williams, V., White, J., 1991. Snake venom variability: methods of study, results and interpretation. Toxicon 29, 1279–1303.

Chippaux, J.P., Boche, J., Courtois, B., 1982. Electrophoretic patterns of the venoms from a litter of *Bitis gabonica* snakes. Toxicon 20, 521–522.

Dimitrovand, G.D., Kankonkar, R.C., 1968. Fractionation of *Vipera russelli* venom by gel filtration—II: comparative study of yellow and white venoms of *Vipera russelli* with special reference to the local necrotizing and lethal actions. Toxicon 5, 283–288.

Johnson, E.K., Kardong, K.V., Ownby, C.L., 1987. Observations on white and yellow venoms from an individual southern Pacific rattlesnake (*Crotalus viridis helleri*). Toxicon 25, 1169–1180.
Kornalik, F., Master, R.W.P., 1964. A comparative examination of yellow and white venoms of *Vipera ammodytes*. Toxicon 2, 109–111.
Mackessy, S.P., 1988. Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus*. Copeia 92–101.
Mackessy, S.P., Williams, K., Ashton, K.G., 2003. Ontogenetic variation in venom composition and diet of *Crotalus oreganus concolor*: a case of venom paedomorphosis? Copeia 769–782.
Mackessy, S.P., 2010. Evolutionary trends in venom composition in the western rattlesnakes (*Crotalus viridis* sensu lato): toxicity vs. tenderizers. Toxicon 55, 1463–1474.
Massey, D.J., Calvete, J.J., Sánchez, E.E., Sanz, L., Richards, K., Curtis, R., Boesen, K., 2012. Venom variability and envenoming severity outcomes of the *Crotalus scutulatus scutulatus* (Mojave rattlesnake) from Southern Arizona. Journal of Proteomics 75, 2576–2587.
Post, Y., Puschhof, J., Beumer, J., Kerkkamp, H.M., de Bakker, M.A., Slagboom, J., de Barbanson, B., Wevers, N.R., Spijkers, X.M., Olivier, T., Kazandjian, T.D., Sainsworth, S., Iglesias, C.L., van de Wetering, W.J., Heinz, M.C., van Ineveld, R.L., van Kleef, R.G.D.M., Begthel, H., Korving, J., Bar-Ephraim, Y.E., Getreuer, W., Rios, A.C., Westerink, R.H.S., Snippert, H.J.G., van Oudenaarden, A., Peters, P.J., Vonk, F.J., Kool, J., Richardson, M.K., Casewell, N.R., Clevers, H., 2020. Snake venom gland organoids. Cell 180, 233–247.
Reynolds, L.J., Hughes, L.L., Dennis, E.A., 1992. Analysis of human synovial fluid phospholipase A_2 on short chain phosphatidylcholine-mixed micelles: development of a spectrophotometric assay suitable for a microtiter plate reader. Anal. Biochem. 204, 190–197.
Rokyta, D.R., Lemmon, A.R., Margres, M.J., Aronow, K., 2012. The venom-gland transcriptome of the eastern diamondback rattlesnake (*Crotalus adamanteus*). BMC Genom. 13, 312.
Saviola, A.J., Pla, D., Sanz, L., Castoe, T.A., Calvete, J.J., Mackessy, S.P., 2015. Comparative venomics of the Prairie Rattlesnake (*Crotalus viridis viridis*) from Colorado: identification of a novel pattern of ontogenetic changes in venom composition and assessment of the immunoreactivity of the commercial antivenom CroFab®. Journal of Proteomics 121, 28–43.
Smith, C.F., Mackessy, S.P., 2016. The effects of hybridization on divergent venom phenotypes: characterization of venom from *Crotalus scutulatus scutulatus* × *Crotalus oreganus helleri* hybrids. Toxicon 120, 110–123.