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Analysis of the Venoms of Four Subspecies of the Western Rattlesnake (Crotalus oreganus)

Natalie Crouch
natalie.crouch7@gmail.com

Stephen P. Mackessy
University of Northern Colorado

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Greeley, Colorado

Analysis of the Venoms of Four Subspecies of the Western Rattlesnake

(*Crotalus oreganus*)

A Thesis Submitted in partial fulfillment for Graduation with Honors Distinction and

the Degree of Bachelor of Science

Natalie Crouch

School of Biological Sciences

College of Natural and Health Sciences

May 14, 2021

Analysis of the Venoms of Four Subspecies of the Western Rattlesnake

(*Crotalus oreganus*)

PREPARED BY: _____

Natalie Crouch

APPROVED BY
THESIS ADVISOR: _____

Stephen P. Mackessy, Ph.D.

HONORS
DEPARTMENT LIAISON: _____

Stephen P. Mackessy, Ph.D.

HONORS DIRECTOR: _____

Loree Crow, M.A.

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(*Crotalus oreganus*)

Natalie Crouch

(Dr. Stephen P. Mackessy)

Abstract

Recently, the benefits venom can have in the discovery and development of different medications to assist in treating a variety of diverse human diseases have been areas of research. In order to develop a better understanding of how to evaluate venoms for potential use as therapeutics, one needs to look at the fundamental composition of venom samples. This project aims at discovering and analyzing the basic components from the venom of four different subspecies of the Western Rattlesnake complex (*Crotalus oreganus* and *C. viridis*). Venom samples collected will be subjected to size exclusion chromatography to separate proteins of different sizes in the venom. After fractionation of venoms, the following assays are conducted on individual fractions to locate specific activities of enzymatic venom proteins: metalloproteinase activity, kallikrein-like and thrombin-like serine proteinase activities, phospholipase A_2 activity, phosphodiesterase activity and L-amino acid oxidase activity. Gel electrophoresis will evaluate the complexity of each fractionated protein peak, and samples will be dialyzed, removing salts, and then lyophilized, allowing for stable storage for later experiments. These tests will compare the basic biochemistry of the samples of venoms from the different subspecies to help expand knowledge on venom chemistry differences between closely related subspecies of rattlesnakes. Snakes use their venoms primarily to obtain food, and results will allow for further evaluation of how their differing venom compositions relate to specific aspects of their ecology, such as use of divergent habitats, potential risks of different predators

and effects on various prey species utilized. These subspecies are separated geographically, and rattlesnakes in general expanded their ranges from south/west to north/central regions, so this project will also reflect how their venoms have evolved over time, as well as revealing those components that have remained static and are shared among all subspecies analyzed. Because envenomation symptoms will vary due to venom composition, these results will also help inform what medical sequelae should be expected from particular populations of rattlesnakes.

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Contents

Introduction

Venom composition can vary based on the snakes' age, sex, and geographic distribution. The age of the snake plays a large role in the composition of its venom. As neonates, many rattlesnakes consume lizards and small rodents, but as adults they tend to consume larger rodents, perhaps necessitating a different venom composition as they age. Juvenile rattlesnake venom is more toxic than adult venoms, while adult venoms often contain much higher levels of metalloproteinases than neonates (Mackessy, 1988). Neurotoxic phospholipases A_2 of juvenile rattlesnakes have more lethal venoms than adult rattlesnakes, as this activity is shown to decrease with age, based on a study of the Central American Rattlesnake *Crotalus simus* (Gutiérrez et al., 1991). Adult venom variation between species are the result of natural selection and evolution acting upon a snake species, resulting in random mutations that lead to greater adaptation to specific conditions and prey utilized in an area. Other factors that have caused the population of snake species to separate are natural changes in the landscape, including deep time events, such as tectonic plate shifting to create mountains, and more recent, sudden events, most importantly human expansion leading to loss of habitat. Adaptations to these differential forces cause populations to change over time until sufficient genetic differentiation results in the formation of genetically distinct populations (subspecies) or even so differentiated as to be categorized as different species. The taxonomic status of certain individual groups are continuously debated in the scientific community as to whether or not to be considered subspecies of the same species, or as distinct species (e.g., Davis et al., 2016).

Review of Literature

There are nearly 3,900 species of snakes known on Earth (Uetz et al., 2020). Snakes occur on most of landmasses as well as many of the oceans and freshwater environments.

Individual groups are separated into different species when they are no longer able to breed due to genetic isolation or if DNA-based studies reveal significant genetic differences. Subspecies are a distinct subpopulation of a species, and they differ from species in that a subspecies cannot exist without the species but can only be expressed in relation to the species. At least two subspecies are needed in order to designate subspecies of a species. Subspecies were created due to recognition of population variants in animals that can still interbreed. Though subspecies have similar genetics, the point of determination between normal variation in the population and genetically different subspecies determination can be hard to distinguish. Due to this determination being a gray area, many scientists have different beliefs on whether or not a group of individuals should be considered a subspecies or not, or if the concept of subspecies is even valid.

Venomous snakes have a complex system of glandular secretions that comprise venom, which is used primarily as a trophic adaptation to obtain food, and secondarily to help defend against potential threats. Venom differs from poison because venom has to be injected, whereas poison needs to be consumed. For example, injection occurs through a snake's fangs, whereas poison is more effective by swallowing a toxic animal, such as a *Taricha* newt. The venom contains a variety of proteins, peptides, carbohydrates, neurotransmitters, amines and other substances that are produced within specific cephalic glands. The venom proteins are synthesized via secretory cells that comprise the bulk of the venom gland. They are synthesized in the rough endoplasmic reticulum, passed through the Golgi apparatus and then transported out of the cell into the gland via granules (Mackessy, 1991). Within a single specimen, there could be up to 100 proteins (including isoforms). Venom is composed of a plethora of enzymes that vary between species and even somewhat between individuals. A rough estimate puts the two families of

Elapidae and Viperidae containing around 19,000-25,000 individual proteins in these venoms (Laustsen, 2018). Snakes have been shown to have control of the release of venom during a bite. Both venom glands, on the right and left, can be independently controlled to release the correct amount of venom for the situation the snake is in (Mackessy, 1988). This allows for snakes to save venom if not all is needed to take down their prey. Enzymes experimented on extensively tend to be ones that cause major pathology within humans or other species. There are around half a dozen common enzyme activity tests that can be performed for compounds that are found in most rattlesnake venom that cause humans pathological problems. Knowing more about these enzymes will aid in learning about the symptoms they cause and how to counteract the enzyme in a medical setting to reduce symptoms and help the individual with eliminating the toxins.

Snake venoms may hold the key to many medical advancements that have already been discovered and potentially many more in the near future. Almost 40 years ago, the first successful medication was made from isolating a toxin found in *Bothrops jararaca* venom (Koh and Kini, 2021). Since then, other toxins within venoms have been carefully studied to create new medicines. Antiplatelet and anticoagulant agents are present in many venoms of rattlesnakes. Both possess qualities to help treat heart attacks, pulmonary embolisms and strokes. Heart attacks are one of the top killers of people in the world. Utilizing metalloproteinase enzymes, serine proteases enzymes or other enzymes could be paired to other medicines, including anti-inflammatories, to help break up the clot before it is able to cause problems in the human body. Though more research is needed in order to perfect the concentration and application, it opens up room for amazing medical advantages in the future.

In addition to breaking up clots in the body, venom proteins show promising solutions for helping fight cancer. In total, 18,094,716 new cases of cancer, excluding non-melanoma skin

cancer, arose in the world in 2020, with 1,970,287 of those cases being from North America (Global Cancer Observatory, 2020). Venom enzymes, or more specifically disintegrins, in the venom show the possibility of inhibiting new blood vessels from being made to supply the tumor cells. This research could stop the growth of tumors due to them being cut off from the body's blood supply (Koh and Klnl, 2021). L-amino acid oxidase in venom could also be used as a cancer treatment causing cancer cells to undergo apoptosis/necroptosis, cell death, due to intrinsic or extrinsic pathways that may be signaled because of hydrogen peroxide byproduct, a reactive oxygen species (Mukherjee, 2015). A peptide, exendin-4, was isolated from the venom of the Gila Monster (*Heloderma suspectum)*, a venomous lizard, and is used to help treat type-2 diabetes. This peptide targets the body's natural insulin-producing pathways and can activate it without glucose. This has resulted in the synthesis of Byetta, a medicine currently used to treat type-2 diabetes. These examples just scratch the surface of what has already been discovered and what potential venom holds for future discoveries.

Wollberg et al. (1988) found that myotoxins have a direct effect on contractibility and the body's continuous movements by affecting the channels that act without our muscular system. The mulgotoxins lead to decrease contractile response when the nerve receptors stimulate the muscle. This can be applied to smooth, muscular, or cardiac muscle tissues. Reduced contractility of these cardiac muscles can lead to a reduced ventricular ejection and lower blood pressure; however, that also means less oxygenated blood is leaving the heart and getting to the body. Dias et al. (2012) performed a study on dogs in Brazil to examine the hemodynamic responses to *Bothrops alternatus* venom and to assess the extent to which they were related to metabolic alterations, cardiac tissue damage, and venom kinetics. The results showed that there was no significant change in blood gas, but there was an increase in plasma lactate

dehydrogenase in the lower venom doses. After four hours, the venom was still detectable but in very low quantities, and the injection showed an increase in the subject's hypotension along with a few other metabolism alterations. This information showed that small doses could alter the body's systems and circulate within the body without hurting or killing the animal that was injected. These experiments allowed for the medical researchers to see that the venom, in small doses, can be safely used without killing the patient. Lastly, Mangiafico et al. (2012) performed a similar trial later that year on dogs and rats. Their experiments consisted of administering an experimental medicine that had a low dose of snake venom present, and this was given to the animals to see the effects the medication would have on the blood pressure. The resulting drug was named LCZ696, which is a mixture of cardiovascular medications and snake venom proteins. LCZ696 is the first to show that drugs mixed with a low dose of venom can still be safe and help lower extremely high blood pressure. More trials are needed to perfect this medicine so that it can be added to medical practice to help quickly and safely reduce the blood pressure of patients, allowing for less stress being placed on the heart, arteries, and veins.

The discovery of anti-venom helped to decrease the death rate from envenomation. Anti-venom works by neutralizing the components of an animal's venom. The ability to make anti-venom relies on the detailed knowledge of the biological makeup of the snake's venom. In certain regions of the world, envenomation from snakes causes high death rates among the people. In one example of the efficacy of antivenom, bites by the South America Rattlesnake (*Crotalus durissus terrificus*), a member of the snake family Viperidae, showed a 71.5% decrease in death rate after the introduction of anti-venom for this snake (Baum et. al. 2019). Research is still under way to discover more novel antivenoms using different approaches including, but not limited to, recombinant antibody formats and toxin-neutralizing proteins

isolated from animals. However, currently the most effective and developed antivenoms are in serum form (Laustsen, 2018). Anti-venoms are made by inoculating experimental animals, most often horses, sheep or goats, with increasing doses of venoms and then harvesting polyclonal antibodies from their serum. These animals produce these antibodies due to being exposed to sublethal doses of a certain type of venom from one species (monovalent antivenom) or multiple snake species (polyvalent antivenom) (Silva and Isbister, 2020). After reaching a high level of antibody titer, the antibodies are purified and used as intact antibodies (IgG) or they are subjected to proteolytic cleavage to produce Fab/Fab$_2$ fragments that can then be given to a patient intravenously (Slagboom et al., 2017).

The snakes used in the present study were *Crotalus* species from the family Viperidae, and they represent species/subspecies of the Western Rattlesnake complex (Figure 1). Viperidae are found mainly in Africa, Asia, and Europe. Viperidae contains around 53 species of rattlesnakes (Westerm et al., 2008). The subfamily Crotalinae is mainly found in Asia and in the Americas. They have a pair of heat-sensitive organs on the anterior part of their head that allow them to locate and hunt warm-blooded creatures at night. They also all have a pair of fangs, which are anteriorly placed in the maxilla of the snake. They are able to rotate their fangs, folding them against the roof of the mouth when they are not in use. Crotaline snakes tend to hibernate during winter months and often bask in the sun during the active season. Oftentimes, snakes are seen to den in large groups and have been observed to travel very long distances to locate a denning site that fits their needs. This natural tendency to hibernate and form dens has been shown to be due to juveniles being able instinctively to follow other snake's chemical trails to find den sites without ever having done it before and without parental guidance (Gienger and Beck, 2011). Adults also show specificity to certain sites and some den sites have over a 90%

return rate of the same individuals the next year, even when individuals were quite far away (Hirth, 1966). After those long winter months, they tend to bask, allowing the sun to help them warm up in order to reactivate their physiological systems.

Due to a reduction in habitat, rattlesnakes are starting to experience changes to their health and reproduction cycles that are outside their normal range; Glissmeyer (1951) showed this phenomenon long ago. Their best estimation is that the reduced number of eggs produced, leading to population declines, results from habitat loss (Glissmeyer, 1951).

The composition of venom is the measure of the chemical makeup of the venom obtained from a snake. It often varies based on the genus and species of the snake. However, many of the more significant parts of the venom are shared between species. Most venoms of Western rattlesnakes have significant amounts of metalloproteinases, serine proteinases, and PLA_2 (Mackessy, 2010). The amount of each enzyme found in the venom sample can help to identify species. Some species will have higher levels of metalloproteinases, which produce severe tissue damage and necrosis; however, species with venoms that are higher in metalloproteinase activity tend to be less toxic (Mackessy, 2010). The relative amounts of toxins also may change with age, even within one species (Mackessy, 1988). For example, Durban et al. (2013) showed that there is a gradual reduction, as the snake gets older, of the expression of serine proteinases and PLA_2, causing the venoms of the neonate snakes to be of higher toxicity than venoms of adults of the same species, as was seen for Pacific Rattlesnakes (Mackessy, 1988).
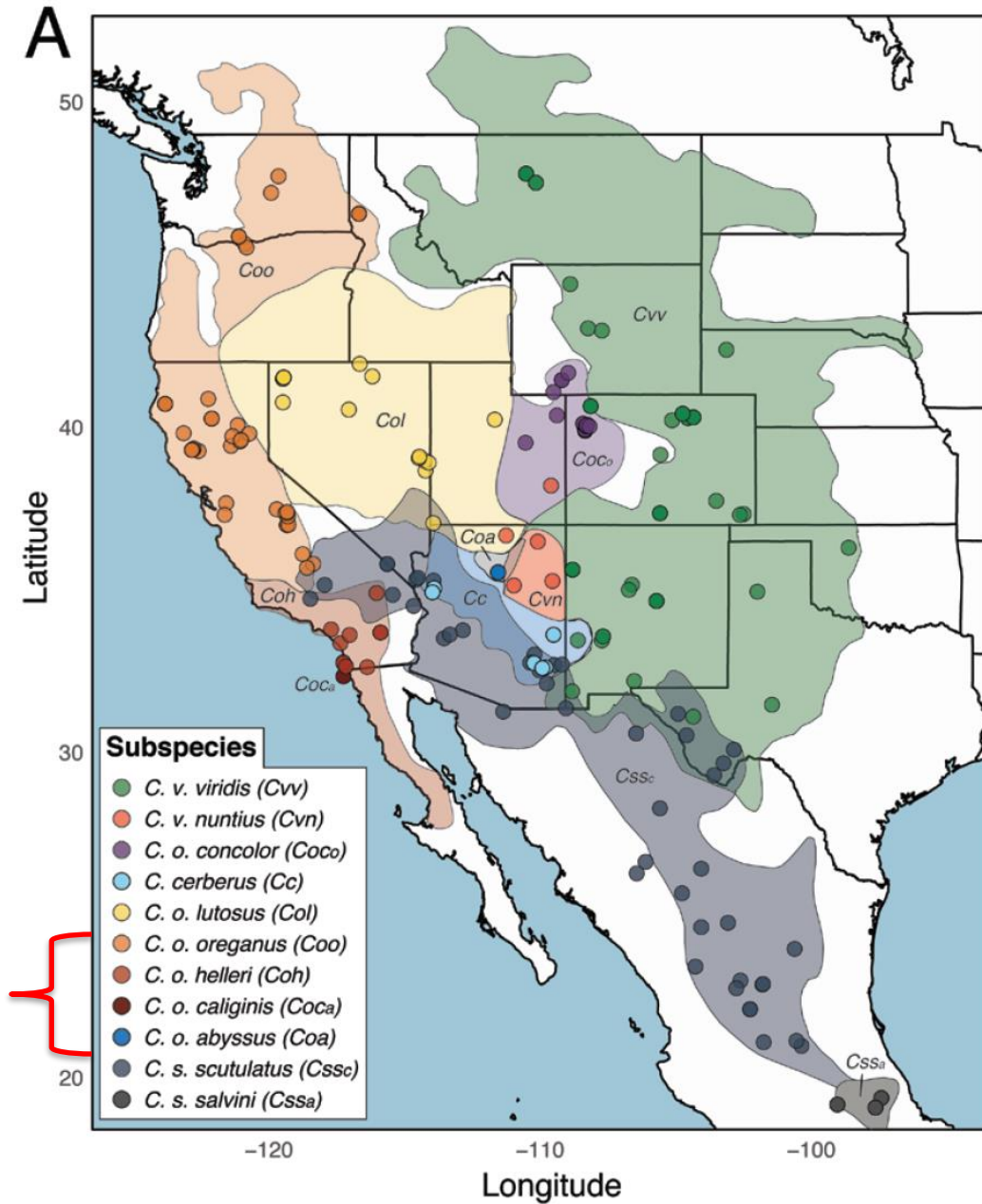
Figure 1. Distribution of the Western Rattlesnake (*Crotalus oreganus* and *C. viridis*) and the

Mohave Rattlesnake (*C. scutulatus*). The four taxa used in this study are indicated by the red

bracket. (Figure is from Schield et al., 2019).

Distribution of snakes used in this study

      Crotalus oreganus helleri, the Southern Pacific Rattlesnake, lives mostly in southwestern

California to northwestern Mexico (Figure 2). Their diet consists largely of lizards as juveniles, including small mammals and birds in adulthood as their venom composition changes with age (Mackessy, 1988; Smith and Mackessy, 2016). The change in diet may be associated with ontogenetic changes in venom composition, and PLA_2 activity decreases while metalloproteinase activity increases in adults as compared to juveniles (Mackessy 1988). Adult Southern Pacific Rattlesnakes show increased proteolytic venom compared to juveniles (Mackessy, 1988; Gren et. al., 2016). The Southern Pacific Rattlesnake tends to be darker brown, gray or black in color.

Figure 2. *Crotalus oreganus helleri* (Southern Pacific Rattlesnake) from Los Angeles; reproduced from Dr. Stephen Mackessy website at the University of Northern Colorado (with permission).

Crotalus oreganus oreganus, the Northern Pacific Rattlesnake (Figure 3), inhabits mostly the Western United States and feeds on small rodents, reptiles and birds based on their age. The

color is extremely variable with light/dark brown/ grey or olive brown. They range in length up to 58 inches (Uetz et al., 2020).

Figure 3. *Crotalus oreganus oreganus* (Northern Pacific Rattlesnake). Photo by S.S. Sweet (1983); reproduced from Dr. Stephen Mackessy website at the University of Northern Colorado (with permission).

Crotalus oreganus lutosus, the Great Basin Rattlesnake (Figure 4), is found mostly in western United States in the Great Basin desert, and they are typically found in more arid environments. They feed primarily on lizards and rodents and have a lighter brown or gray color with darker brown blotches.

Figure 4. *Crotalus oreganus lutosus* (Great Basin Rattlesnake); reproduced from Dr. Stephen

Mackessy website at the University of Northern Colorado (with permission).

Crotalus oreganus cerberus- Arizona Black Rattlesnake

  Crotalus oreganus cerberus, the Arizona Black Rattlesnake, has a darker pattern of either

black, gray, olive or brown with light blotches throughout its body; older males tend to become

nearly completely black (Figure 5). The species is found in southern Arizona to central New

Mexico.

Figure 5. *Crotalus oreganus cerberus* (Arizona Black Rattlesnake); reproduced from Dr. Stephen

Mackessy website at the University of Northern Colorado (with permission).

Assay background

Metalloproteinase assays measure the amount of metalloproteinases in the venom.

Metalloproteinases break down collagen and other proteins in the body when injected from a

snake bite, found as connective tissues in blood vessels, organ structures, basement membranes

of epithelial tissues and extracellular spaces of the body of vertebrates. These venom proteins

cause major structural damage, while other venom components inhibit coagulant factors or

platelets, neutralizing clotting proteins and causing the bite site to be unable to clot and stop the

bleeding. This can cause bleeding at the site of the bite as well as systemic bleeding if medical

attention is not received immediately. Metalloproteinases also disrupt vascular endothelium that

is found on the inner lining of veins, capillaries, and arteries, allowing blood to escape through these passages as a result of damage to the walls. Due to this anticoagulation and vascular endothelium, thinning the metalloproteinases helps to spread other proteinase components into the circulatory system of the victim (Maruyama, 2013). Most rattlesnakes venoms contain high metalloproteinase activity and bites can lead to severe bleeding and potential death if left untreated.

Serine proteases (both thrombin-like and kallikrein-like) aid metalloproteinases in the disruption of the vascular system and the disruption of the body's defense to clot or repair blood vessels. L-amino acid oxidase is responsible for many different side effects from snake venom bites and is very common in venomous snakes. The enzyme is a homodimer consisting of three domains: substrate-binding domain, FAD-binding domain, and helical domain (Doley and Kini, 2009). The L-amino acid oxidase gives rise to the yellow color of venom due to the presence of the cofactor flavin adenine dinucleotype (FAD). The enzyme's activity gives rise to peroxide radicals that are able to damage various structures, including DNA. Though some biological effects are unclear, and it is uncertain exactly how L-amino acid oxidase affects humans, it is believed to activate inflammation and promote a pro-apoptosis factor in human cells, primarily as a result of generation of peroxide radicals (Watt et al., 2004).

Phospholipase A_2 exists in significant amounts in many snake venoms, especially in younger snakes, and may act as a neurotoxin. They have been found to be monomers but can interact with other phospholipase A_2 or other proteins. Isoforms of phospholipase AA_2have been shown to bind to the presynaptic site of a neuromuscular junction and cause acetylcholine to not be released as readily. This decrease of acetylcholine can lead to flaccid paralysis of the muscles and shut down of neurotransmission (Doley and Kini, 2009). Other non-neurotoxic

phospholipase A_2 can enter the circulatory system and cause heart problems, including hypotension. It also may interact with other enzymes in the venom to inhibit platelet formation, promote anticoagulation and can cause inflammation at the site of the injection, as well as systemically (Gimenes et al. 2014).

Phosphodiesterase activity can disrupt cell signaling in the body as well as act to hydrolyze both RNA and DNA. Phosphodiesterase works best under somewhat basic pH and catalyzes the hydrolysis of phosphodiester bonds of mononucleotides (Dhanajaya and D'Souza, 2009). In the human body, these enzymes cause pathology at ruptured blood vessels, skin necrosis, deep tissue damage, osteomyelitis, and other significant consequences (Warrell, 2010). The inability of the snakebite wound to clot also increases the risk of secondary infection due to an open wound being exposed to the external environment. Due to many snake bites happening in the environment, oftentimes not close to civilization, many other pathogens tend to be present at the location that the bite occurred. Snakebite pathology can cause localized problems, including pain and edema or more systematic effects including chronic conditions (Slagboom et al., 2017). Symptoms vary based on numerous factors, including the snake species that caused the bite, the amount of venom injected, the location of the bite and how long it takes to get medical treatment.

Materials

All protein reagents were purchased from BioRad Inc. (Hercules, CA, USA) and NuPage gels were obtained from Life Technologies, Inc (Grand Island, NY, USA). All other reagents were purchased from Sigma Biochemical Corp. (St. Louis, MO, USA). Size exclusion column, incubators, water bath, Agilent spectrophotometer and all other testing equipment were supplied

in the venom lab at the University of Northern Colorado by Dr. Stephen Mackessy.

All venoms were obtained from captive snakes maintained in the Animal Resource Facility in Ross Hall at UNC. Venoms were extracted manually as described previously (Mackessy, 1988) and then typically lyophilized and stored frozen until needed.

Methods

Extractions

The first step to venom analysis is to collect the venom. This can be done from lyophilized venom previously collected or from fresh extraction of venom from the snake. In order to extract venom from a rattlesnake, precautions need to be practiced, included but not limited to not extracting the venom without Dr. Mackessy present (who is the only one extracting venoms), utilizing tools and safety techniques to obtaining the snake out of the cage, and clearing an area to make sure no one is in harm's way. Once the snake is safely obtained, the snake is put under anesthesia using isoflurane in O_2 in a small restraint box and once anesthetized, the snake is held by the back of the head to ensure no risk of a bite. Then, using small capillary tubes, an assistant places the tubes on each fang, being careful not to tear the delicate skin of the mouth, and the venom glands are gently massaged to release the fangs without hurting the snake. Once the venom is extracted, the amount is measured and recorded; additionally data on the individual snake is recorded.

Size Exclusion Chromatography

Size exclusion chromatography utilizes a column filled with porous beads that are made of polyacrylamide and the buffer solution. The beads have pores of varying sizes that help sort

macromolecules into different size classes as a sample moves through the column, and the small macromolecules are slowed by passing through most pores of the beads. Larger macromolecules emerge first and as time goes on, the smaller macromolecules come out. Size exclusion columns can be calibrated to determine the size of the proteins that fall within a certain size range. Once the size exclusion chromatography is finished running, the fractionated venom collected into tubes (termed fractions) is measured at 280 nm. The data is then plotted as fraction number vs. absorbance at 280 nm. Fractionated venom in the tubes is then sampled and diluted with a buffer specific to a particular enzyme assay.

Crotalus oreganus lutosus size exclusion was done with venom from one male and one female snake; 100 µL from each were mixed with 800 µL of 25 nM HEPES, 100 nM NaCl, 5 nM $CaCl_2$ (pH 6.8) and run at flow rate of 6.0 mL/hr. Both snakes were extracted from the UNC collections of snakes (male was 2019-44 extraction and female was 2019-478). It was assumed to be 50 mg of proteins loaded, as rattlesnake venoms contain approximately 225-250 mg per mL protein.

Crotalus oreganus oreganus size exclusion was a mixture of venom collected from a Lewiston, Idaho den site on April 17, 2019. 50.7 mg was dissolved in 750 µL 25 nM HEPES, 100 nM NaCl, 5 nM $CaCl_2$ (pH 6.8) buffer 12.6 mg of C. o. oreganus: 2019.228, DRS:346, ID: Nez, mixed with 12.718 mg of *C. o. Oreganu*s: 2019.231, DRS: 349, ID: Nez, with 12.882mg *C. o. Oreganus:* 2019.224, DRS: 342, ID: Nez, with 12.448 mg of *C. o. oreganus*: 2019.226, DRS:346, ID:Nez.

Crotalus oreganus oreganus size exclusion was obtained from an individual extracted on April 23rd 1984 from the Carrizo Plain, California. 21.2 mg of solid venom was mixed with 750 µL of 25 nM HEPES, 100 nM NaCl, 5 nM $CaCl_2$ (pH 6.8) buffer.

Crotalus oreganus helleri venom was a mixture of two individuals from Los Angeles Co., California in 2018. 60.1 mg of *C. o. helleri* (2018-208) was dissolved in 1500 µL of 25 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$ (pH 6.8) buffer.

Crotalus oreganus cerberus size exclusion used venom taken from individual 491 (2020-172). 53.1 mg lyophilized venom was dissolved in 750 µL of 25 nM HEPES, 100 nM NaCl, 5 nM $CaCl_2$ (pH 6.8) buffer.

Assays were then run on fractions to test for the presence of particular activities within the venom. This is done by taking a small aliquot of fractionated venom, combining it with a substrate specific for an enzyme that causes a reaction resulting in a color change that can be measured to obtain quantitative data. Each assay tests for a different enzyme (known to be most common in rattlesnake venoms), allowing for detection and quantification of different venom enzyme components that are known to cause major pathology for animals (including humans) when a bite from a rattlesnake occurs.

Azocasein metalloproteinases assay (AZO)

Azocasein metalloproteinase assay followed a modified method of Aird and da Silva (1991). Metalloproteinases are larger macromolecules that resides in the first few peaks of venom that has gone through a size exclusion tube. The assay is done in 50 mM HEPES, 100 mM NaCl (p.H. 8.0) buffer and azocasein protease substrate dissolved in the buffer at a 2.0 mg/mL. 35 µL of column fraction with 365 µL of buffer and 35 µL substrate/buffer mix is used, terminated after 30 min with of 250 µL 0.5 trichloroacetic acid stop solution. Insoluble material is then pelleted via centrifugation, and the supernatant is then read at 342 nm.

Thrombin-like and kallikrein-like serine protease assay (THR and KAL)

Thrombin-like serine protease assay and kallikrein-like serine proteinase assays were based on modifications of Mackessy (1993). Both assays are done in the separate test, but both use 370 µL of the buffer of 50 mM HEPES with 100 mM NaCl at p.H. 8.0 with 5 uL of column fractions. However, kallikrein-like assay uses 50 µL of kal substrate where thrombin-like uses *50 µL of* THR substrate. All tubes were incubated at 37 C for 8 minutes total, 3 without substrate (to bring to temperature) and then 5.0 minutes after the substrate was added. Both reactions are terminated with 75 µL of 50% (v/v) acetic acid. The experiment is then read at 405 nm with two test blanks of 5 µL water instead of venom.

L-amino acid oxidase assay (LAAO)

L-amino acid oxidase procedure was modified from Kishimoto and Takashahi (2001). L-amino acid oxidase is a plate-based assay that combines 10 µL of venom from each fraction with 90 µL of a master mix. The Master mix contains 10X concentrations of L-methionine, o-phenylenediamine and horseradish peroxidase in the borax buffer. In order to get each reagent to a 10x concentration they are mixed in solid form with a buffer. 5.0 mM L-methionine is combined with borax at a 7.46 mg per 1 mL ration, 2.0 mM o-phenylenediamine is combined with buffer at 3.246 mg per 1 mL per 1mL ratio with buffer, and lastly horseradish peroxidase is combined with buffer at a o.316 per 1mL ratio to make a 100x stock solution. Horseradish peroxidase is then diluted in more buffers to make a 10x concentration that can now be used for the master mix. Per 1 mL, 700 µL of 50 mM borax buffer (pH 8.5) is combined with 100 µ*L* each of L-methionine, o-phenylenediamine and horseradish peroxidase. After venom fractions and the master mix are combined, the procedure outlined to incubate the plate for 15

minutes at 37 C; however, all tests were terminated after 5 minutes due to high activity levels. 50 µL 2.0 M sulfuric acid was added to terminate the reaction and the plate was read at 492nm with two blanks of 10*uL* of water instead of venom.

Phospholipase A$_2$ activity (PLA$_2$)

Phospholipase A_2assay was modified from Holzer and Mackessy (1996). The procedure was modified to be a plate based assay. This was done by adding 165 µL *of* buffer that was made of 10mM tris-HCl, pH 8.0) with 10 nM Ca$Cl_2$and 100 nM NaCl, to 10 µL *of* venom. Then 25 µL *of* 3.0mM 4-nitro-3-(octanoyloxy) benzoic acid was added after being diluted in acetonitrile at a ratio of 0.96 mg per mL. The plate was incubated for 60 minutes at 37∘ C and then read at 425nm frequency with two blanks of water instead of venom. *Crotalus oreganus oreganus* (present day) and *C. o helleri* venom fraction amount and substrate amount were increased (to 40 µL) due to not seeing any significant data when procedures were run.

Phosphodiesterase

Phosphodiesterase activity was done based on Laskowski (1980) modification of Bjork (1963). The biggest modification was it was a changed to be a plate based assay. 20 µL of venom from a fraction was mixed with 70 µL of 100 mM tris-HCl (pH of 9.0) with 10 mM MgCl_2. Then the substrate is made by 50 µL of 1.0 mM bis-p-nitrophenyl phosphate was dissolved in a buffer at a 0.362 mg per 1 mL concentration. Once substrate was added it was incubated at 37∘ $C$for 30 minutes and then immediately 125 µL of 100 nM NaOH with 20 nM diNa-EDTA to terminate the reaction. The plate is read at 400 nm absorbance with two blanks of 20 µL of water instead of venom.

Storing venom

The fractions need to be kept refrigerated in order to keep the venom in a working state. However, keeping around 200 test tubes of venom for each snake species takes up a lot of room and therefore once tests have been performed, the first 30 test tubes (ones with zero absorbance for initial absorbance test) can be discarded due to consisting of only buffer. After assays are completed, fractions making up specific peaks can be combined. Once combined, a gel can be run on a venom sample (compared to standards) to examine the amount of protein present in the venom and sizes of the protein for the whole venom compared to a different proteins in each size exclusion peak that has been combined. After a gel has been run, the gel needs to be stained and then excess stain needs to be taken up so the gel lines are viable. Gels were not run on all species, only *C. o. lutosus* and *C. o. oreganus*. Once a gel is complete, the different peaks can be dialyzed using dialysis tubing to remove salts from the venom/buffer solutions. After 24 hr, the dialysis water was changed to maintain the salt concentration being higher in the venom solution compared to the water. Then the different combined peaks can be lyophilized, for smaller and easier storing that will keep the venom stable for later use on experiments. If the venom is needed for later experimentation, the sample is then rehydrated.
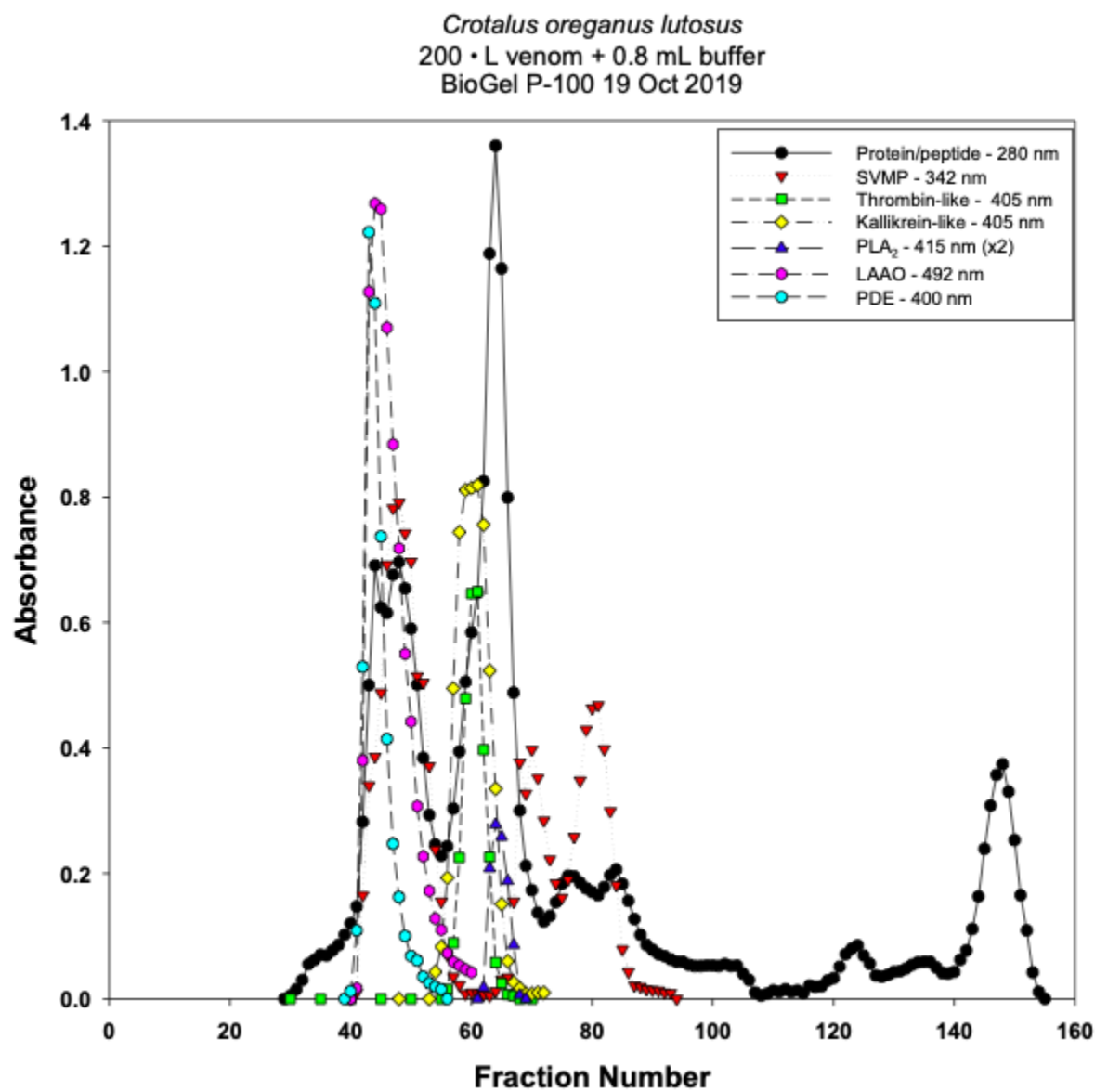
Data analysis

Figure 6. Size exclusion chromatogram of *Crotalus oreganus lutosus* venom. Approximately 50

mg venom from 1 male and 1 female snake were mixed with 800 µL of 25 mM HEPES, 100 mM

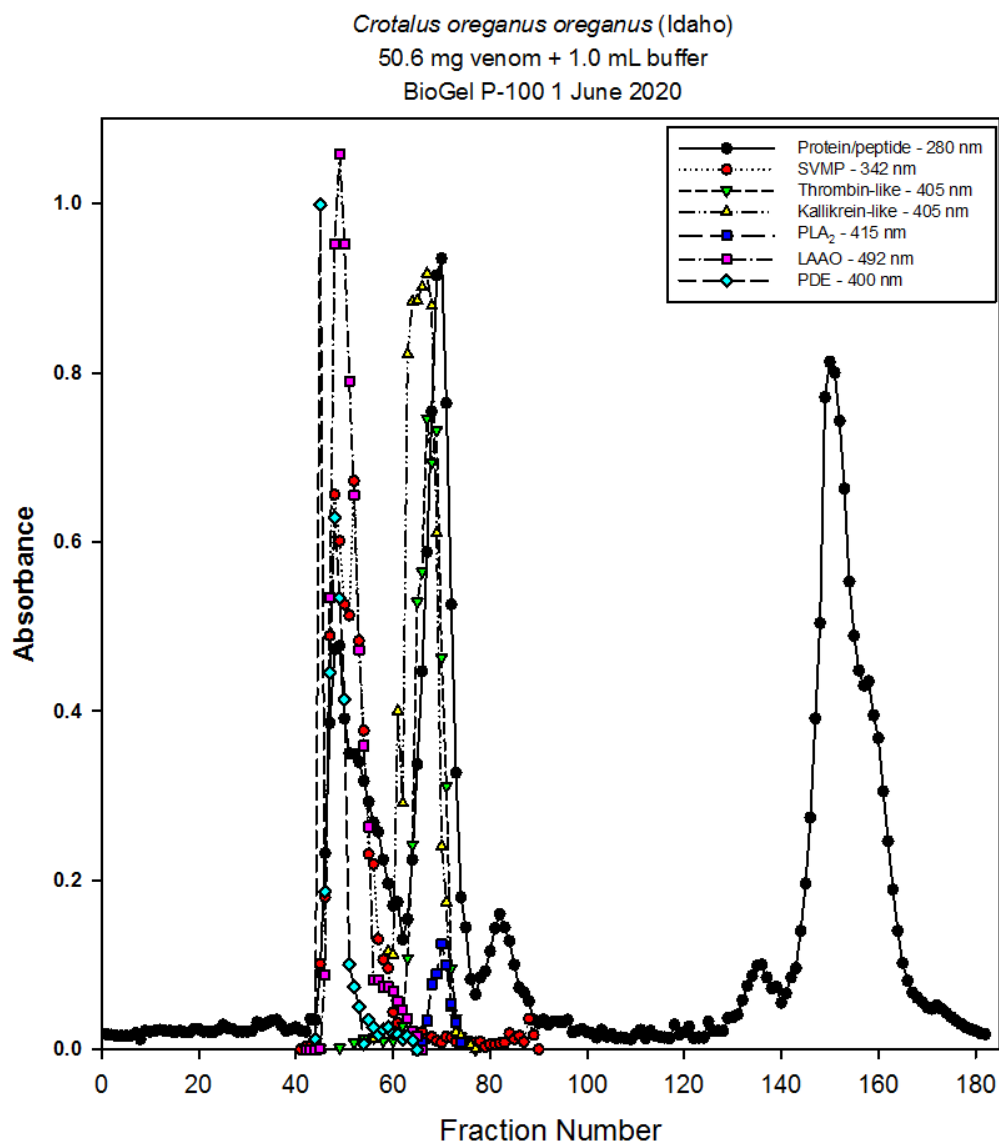NaCl, 5mM CaCl$_2$ (pH 6.8) and run at flow rate of 6.0 mL/hr.

Figure 7. Size exclusion chromatogram of *Crotalus oreganus oreganus* venom. Venom was collected from snakes from a Lewiston, Idaho den site. 50.668 mg venom in 1.0 mL, with a flow rate of 6 mL/hr.
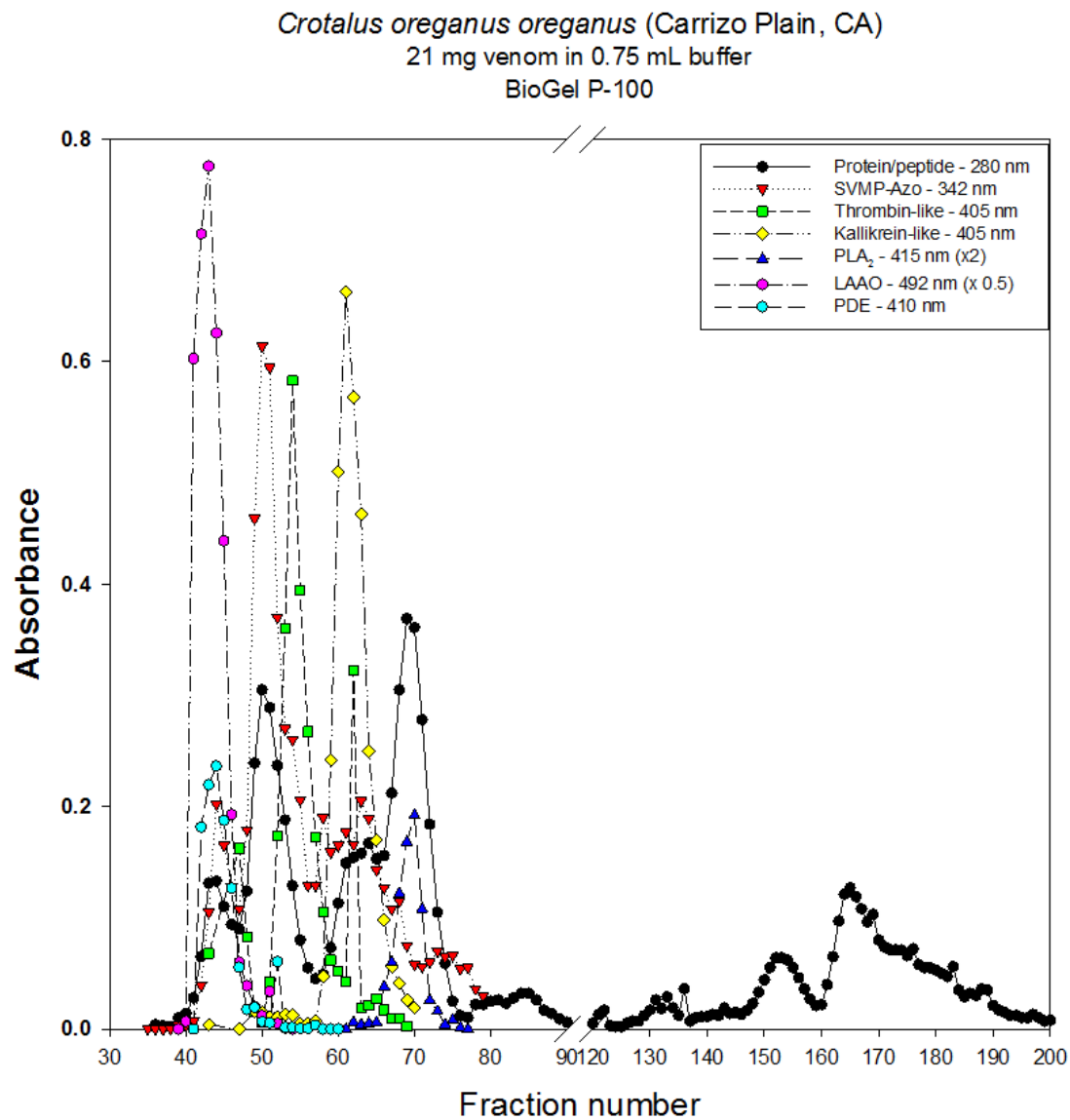
Figure 8. Size exclusion chromatogram of *Crotalus oreganus oreganus* venom. Venom was collected from an individual extracted on April 23rd 1984 in Carrizo plain California. 21.2 mg of venom was mixed with 750 µL of 35mL, with a flow rate of 6mL/hr.
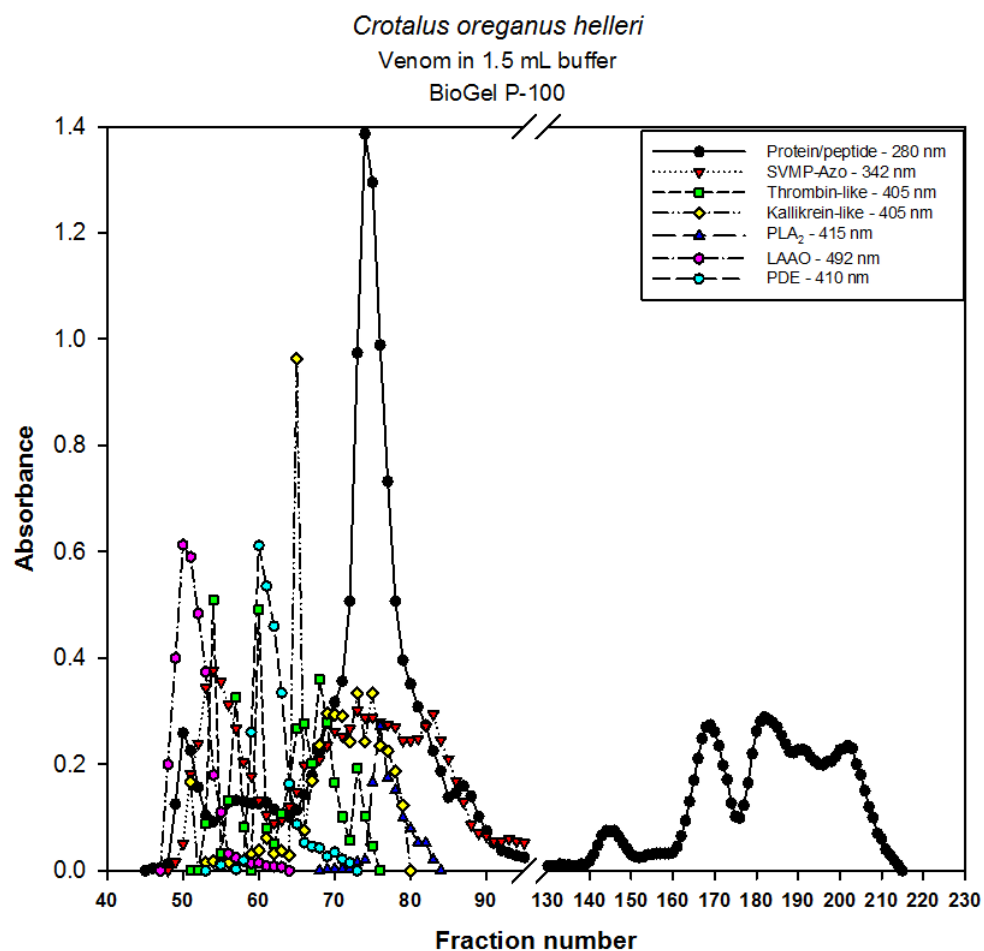
Figure 9. Size exclusion chromatogram of *Crotalus oreganus helleri* venom. The venom was a mixture of two individuals from Los Angeles California in 2018. 60.1 mg of *C. o. helleri*: 2018.208 was mixed with 1500 µL of buffer.
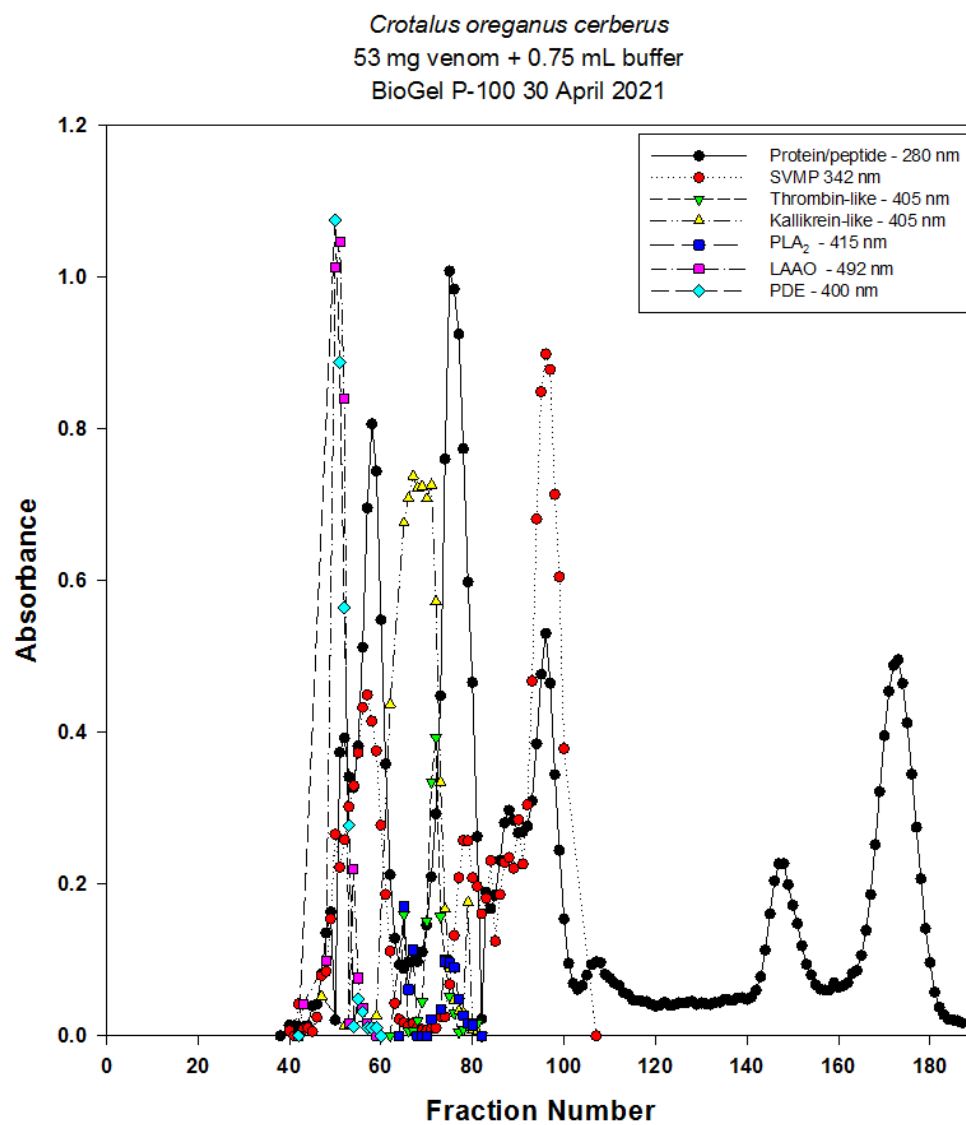
Figure 10. Size exclusion chromatogram of *Crotalus oreganus cerberus* venom. Venom was taken from individual 491, from 2020.172. 53.14 mg solid venom mixed with 750 µL, with a flow rate of 6mL/hr.
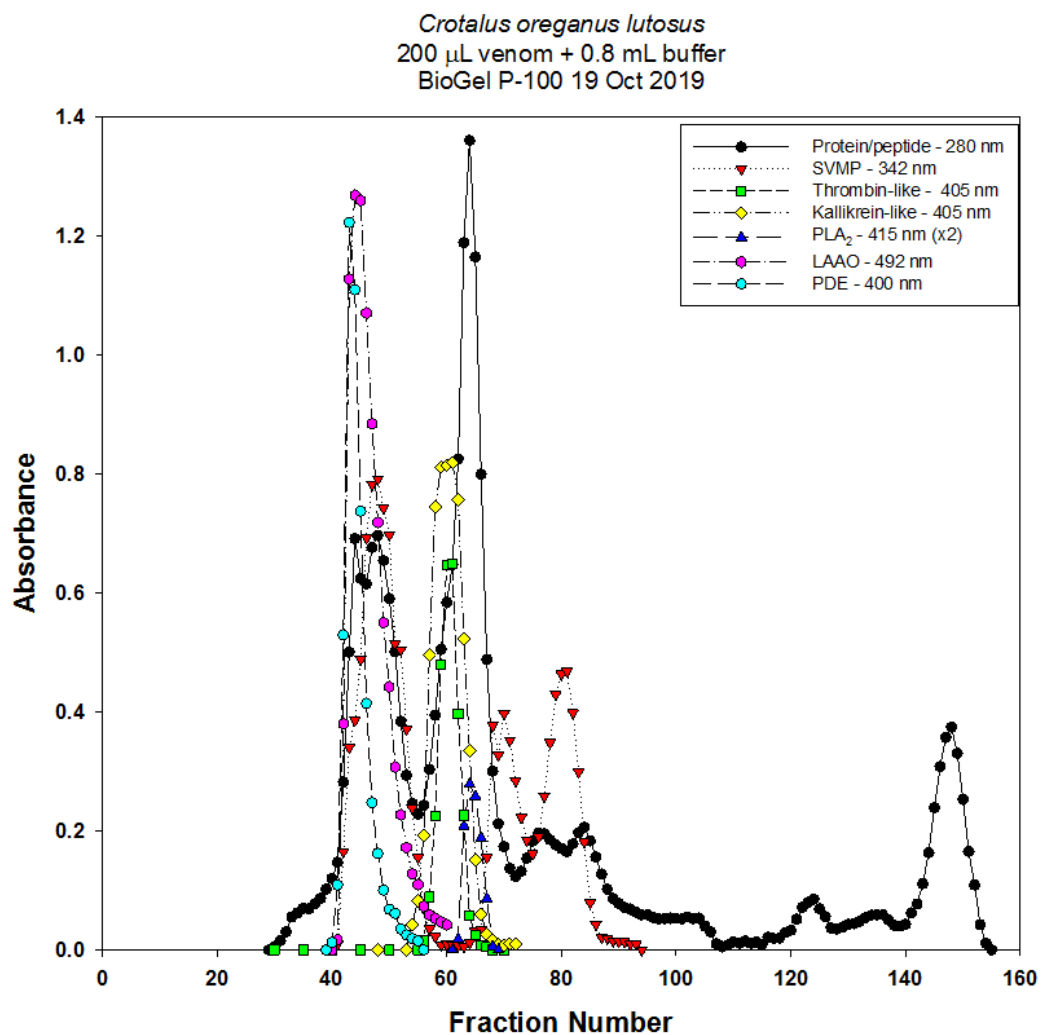
Figure 11: Size exclusion chromatogram of *Crotalus oreganus lutosus* venom. Venom was taken from a male and female mixed together at a 50:50 ration of 100 µL each mixed with 800 µL of buffer.

Discussion

Examining all five subspecies shows that LAAO and PDE enzymes are the largest size proteins in each venom. Both assays revealed them to be present in the first peak of activity after the size exclusion fractions were tested. Kallikrein-like activity and PLA_2 were consistently seen to be in the second peak (or slightly before the second peak) in all individuals. The remaining assays showed up in multiple different places within the first two-three peaks; this includes thrombin-like as well as metalloproteinase activities. This is due to these enzymes having multiple sized proteins of these enzymes within the venom. After elution of enzymes (which are larger), the background absorbance remained at blank levels until the smaller peptides eluted late in the chromatogram. Levels of these peptides were variable between samples.

Size exclusion fraction readings varied greatly between species. Both individuals of *C. o. oreganus* and the *C. o. lutosus* species showed two large peaks within the first 40-100 fractions. *Crotalus oreganus helleri* displayed one large peak in the same range, as well as a smaller, less significant peak proceeding the large peak. Lastly, *C. o. cerberus* differed in size exclusion due to having three significant peaks between fraction numbers 40-100. After the beginning peaks, all fractions dropped to around zero until around fraction reading 120, where all individuals had low mass downstream peaks. These peaks ranged from one peak to multiple peaks. The height of the peaks ranged from the smallest 0.369 $A_{280\,nm}$ (*C. o. oreganus* Carrizo) to 1.386 (*C. o. helleri*), followed closely by 1.36 in *C. o. lutosus*. *Crotalus o. oreganus* (Carrizo) had the lowest peak due to being the lowest total venom protein load.

Metalloproteinase activity showed variation from all other individuals due to having three significant peaks in *C. o. lutosus* within the first 100 fractions compared to all other individuals having one-two peaks in the remaining individuals. All peaks ranged from 0.377 in Helleri to 0.89885 in Cerberus with all remaining individuals between those two. Both *C. o. oreganus*

individuals*, C. o. lutosus* and *C. o. cerberus* had higher levels (0.614-0.899 absorbance) compared to *C. o. helleri* (0.377), indicating that *C. o. helleri* may not need metalloproteinase activity to be as high for prey along the southern California coast.

All thrombin-like and kallikrein-like serine protease activity is most dominant in the second peak on the size exclusion reading with most species having higher levels of kallikrein compared to thrombin. Multiple individuals, including *C. o. helleri* and *C. o. oreganus*, had two peaks for thrombin within the first 40-100 fractions numbers. Kallikrein was seen to have higher activity than thrombin in all individuals except in *C. o. lutosus*, where the peaks were not in the same place, where both levels were exactly the same at a max peak at 0.649 absorbance. *Crotalus oreganus helleri* was seen to have the highest peak level of kallikrein at 0.963 absorbance and was also seen to have the one of the lowest thrombin activity at 0.510 absorbance. All individuals had between a 0.565-0.832 average absorbance when adding thrombin and kallikrein activity together divided by two. All samples that have higher levels of kallikrein tended to have lower levels of thrombin, with *C. o. lutosus* being in the middle with their activity being the same. This pattern shows the possibility that as one activity level increases (thrombin or kallikrein), the other activity seems to decrease. Kallikrein had the highest levels in *C. o. helleri* and *C. o. cerberus* as well as the two lowest levels of thrombin enzyme active. Both subspecies are located in the southern part of the United States, showing location may affect levels of serine protease activity.

Phospholipase A_2 activity was low for all five samples run, with readings ranging from 0.096-0.170 absorbance. In order to visualize activity on some of the graphs, readings were multiplied by 2 (shown in legend as X2). *Crotalus oreganus cerberus* was the only individual to show two peaks of PLA_2 activity, compared to one peak in all the other four individuals. *C. o.*

cerberus also had the highest activity at 0.17. Though all individuals were close in range, reasons for some variation in age of the snakes, since PLA$_2$ decreases with age.

All four species and all five individuals have high levels of LAAO activity; from the literature, LAAO is very common in many species and as size exclusion was run on each individual, all had an area of fractions with slight yellow tint, showing evidence of LAAO. *Crotalus oreganus cerberus* and *C. o. oreganus* (2019) had slightly lower activity than all other individuals, with a peak at 1.058 (*C. o. oreganus*) and 1.047 (*C. o. cerberus*) compared to 1.225-1.553 in the other individuals.

Phosphodiesterase activity was most significant in the first peak of each fraction in all individuals. The lowest activity was shown in *C. o. oreganus,* the rest of the individuals fluctuated significantly from 0.237 in the Carrizo *C. o. oreganu*s compared to 1.0751 absorbance reading in *C. o. cerberus*. *Crotalus. oreganus. cerberus* and *C. o. heller*i showed the highest level of PDE compared to the other species and both *C. o. cerberus* and *C. o. helleri* tend to live closer to the southern United States than the rest of the species. The climate and prey selection in the more southern states may play a role in the increased level of PDE.

Data from the *C. o. oreganus* individuals taken in 2019 compared to the individuals in 1984 showed significant differences. The snake in 1984 (Carrizo) showed two separate sized thrombin-like enzyme activity peaks and where the snake from 2019 did not. The 2019 snake, however, had high levels of thrombin at a peak of 0.746 compared to 0.583 as well as showed higher levels of kallikrein activity at 0.917 compared to the 1984 snake only peaking at 0.663. The 1984 individuals also showed significantly higher PDE levels peaking at 0.9985 absorbance compared to the 2019 individual only reaching a peak of 0.2365. However, the 2019 snake had larger LAAO activity with a peak spiking at 1.553 compared to 1.058 in the 1984 individuals.

Overall, AZO activity was similar in both species: 0.672 in the 1984 individual compared to 0.614 in the 2019 snake. As well as PLA$_2$ activity of 0.096 peak in the 1984 compared to 0.125 in the 2019 snake. Snakes, like humans, are all unique but this significant amount of differentiation in their composition of venom shows genetic effects between these two species beyond just being individuals. The difference could be due to location, time of isolation or prey selection differing between the area the two individuals reside. To determine what caused the significant difference in the two species, future research would need to be done.

All five individuals displayed slightly different activity levels of each enzyme but all had activity for each assay run. This goes to show that natural selection, genetic drift, and other factors acting on random mutations have caused these subspecies to adapt to their new environment and cause their venom component to slightly change activity. There were notable differences in certain assay based on the year the venom was taken and some assays show the possibility of location playing a factor into enzyme active differences. All subspecies give scientific proof of the similarities and difference between these subspecies venom components. Phospholipase A$_2$ being in low quantities for these individuals is due to all test runs being run on adult rattlesnakes. Phospholipase A$_2$ tend to decrease in activity as the juveniles are seen to have significantly greater amounts than adults. *Crotalus oreganus lutosus* showed the highest level of venom activity compared to all other individuals. In order to get a clearer comparison between the individuals of each subspecies, having the same age snakes would help to eliminate age bias in enzyme activity levels.

The frontier of snake venom in medicine and scientific research is just on the brink of explosion. The applications of venom in all fields is an exciting development that can change the way we treat life threatening diseases including but not limited to cancer, heart attacks and

strokes. The possibilities are still unknown for the application of these potentially life-saving components as the gap in turning venom into medicine is unstudied. Researchers need to see what the appropriate dose is to have enough venom to help but not too much that it hurts the patient. The future studies also need to address where in the body the venom proteins target wherein and if the researcher can use them to make medicine that helps though body systems without hurting the patient. Could it one day be injected to cure someone you love?

Additional future research that can be done using the data above includes categorizing venom from isolating the proteins that are now in known locations. Once the venom is isolated, the components can be used in medical studies to help create new medications or the information on the levels of enzymes can be used for creating new anti-venoms. Determining patterns of levels of each enzyme in these subspecies also gives rise to information for determining evolutionary compounds. Seeing that different levels of activity that are present in different species can then be compared to diet preferences, location and other factors to see what aspects of natural selection are acting on mutation in venom to have driven these subspecies to have different levels of each enzyme.

Potential sources of error

Differences in size exclusion column performance could also lead to errors. This was observed as diffuse peaks instead of well-defined peaks (not a major issue for this study). Inaccurate disruptions can happen through contamination or old matrix beads that are not collapsed and non-porous. *Crotalus oreganus helleri* venom was run on a different size column bead (fine, instead of medium), and the smaller beads caused the run to go longer, but it was accidentally stopped early. Because these later peaks did not pertain to the data used, the test was

not redone for *C. o. helleri*. Other sources of error are human errors including pipetting errors, accidentally doubling or forgetting to add substrate to tubes, and double absorbance reading of tubes.

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