Development and Identification of Affibody Molecules that Target Crotalid Snake Venoms.

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ABSTRACT

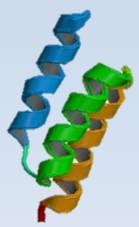
Affibodies are a type of small protein that can be designed to bind with high affinity to a researcher's protein of choice. They have been utilized in a variety of different applications, including therapeutics, in vivo imaging, and biotechnological applications. Affibody molecules that are specific to a protein target can be selected for using a library of affibody molecules displayed on phage. In this study, a library of affibody molecules displayed on M13 bacteriophage was used to probe affibody/snake venom interactions. This particular library was developed by New England Biolabs and was used to pan against Western diamond back rattlesnake (*Crotalus atrox*) and Western cottonmouth (*Agkistrodon piscivorus leucostoma*) venoms. By performing the panning procedure of the affibody phage display library against snake venom, we hope to determine which affibodies have an optimal affinity for the venoms. By identifying affibody molecules that have affinity for snake venom proteins, we hope to perform further protein/protein interaction studies that will contribute to the field of venom research.

PURPOSE

Each year in the United States, 7000-8000 people are bitten by venomous snakes, which is a physically painful and financially costly experience which may even lead to death. The Crotalinae subfamily of pit vipers, which includes rattlesnakes, copperheads, and water moccasins, are commonly found in many parts of the U. S., including Texas. CroFab, the current antivenom for pit viper snake bites in the U. S., is derived from antibody fragments harvested from sheep and effectively treats most snake bites, but there are some limitations to the antivenom. In order to expand our knowledge of snake venoms and possibly open the door to new antivenom options, an affibody phage display library will be used to develop and identify affibody molecules that specifically target Crotalid snake venom proteins. In this project, we will be using an affibody phage display library developed by New England Biolabs to pan against Western diamondback rattlesnake (Crotalus atrox) venom.

AFFIBODY

Affibodies are randomized proteins based on the Z-domain of *S. aureus* Protein A and are widely used in phage display experiments to select subnanomolar binders to various targets. Their structure is comprised of a three-helix bundle of 58 amino acids, with a molecular weight of 6 kDa.



An example of an affibody crystal structure. This in particular is the ZHER2 Affibody.

http://www.rcsb.org/pdb/explore.do?structureId=2KZJ

G6813A AFFIBODY PHAGE DISPLAY LIBRARY

The displaying of proteins or peptides on the phage surfaces, or phage display, has been used for a variety of different applications ranging from drug development[1] to materials science.[2] Because of the link between the phage DNA and the protein encoded on its surface, phage display is a powerful screening tool. Here, we propose to use this tool as method for studying affibody/snake venom interactions. Affibody molecule libraries attached to phage have also been used in other applications.[3]

USER technology (NEB) was used to construct an Affibody library of $1x10^9$ complexity using a variant of the phage display cloning vector M13KE (NEB) with a G6813A mutation (for genetic stability) in the 5' UTR of gII, and a synthetic insert containing the affibody gene with 13 randomized codons (each NNK, where N is equimolar G,A,T,C; and K is equimolar G,T). Through this method, a partially randomized DNA segment encoding the affibody protein was added to the N-terminus of gene III, which encodes pIII.

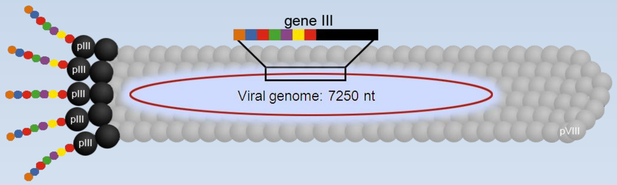
SNAKE VENOM PROTEINS

The venom used in our experiment was isolated from the Western Diamondback Rattlesnake, *Crotalus atrox*, a crotalid commonly found in Texas. The proteome of this venom includes 69.5% snake venom metalloproteinases (SVMP) and serine proteinases, 25.8% medium-size disintegrin, PLA(2), cysteine-rich secretory protein, and l-amino acid oxidase, and 4.7% vasoactive peptides, endogenous inhibitor of SVMP, and C-type lectin-like proteins. This composition potentially explains the cytotoxic, myotoxic, hemotoxic, and hemorrhagic effects evoked by *C. atrox* envenomation.[4] This snake venom from this species was purchased from the National Natural Toxins Research Center at Texas A&M Kingsville.

C. atrox: The western diamondback rattlesnake.
http://www.joelsartore.com/galleries/greatest-hits/43/

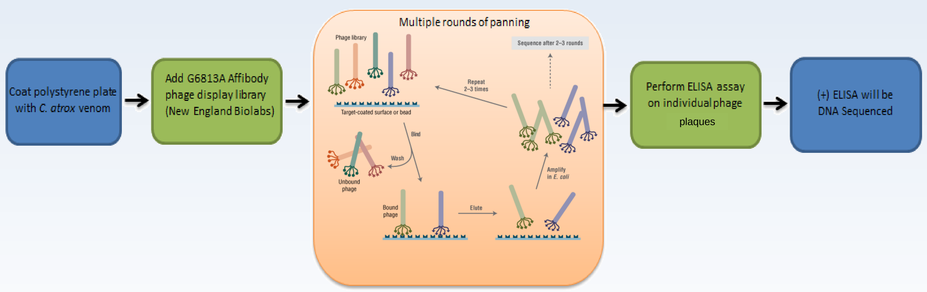
BACTERIOPHAGE

The filamentious bacteriophage M13 is a virus that infects bacteria. A bacteriophage is composed of a single-stranded circular DNA genome surrounded by a protein coat that serves to protect it and also to interact with its bacterial cell targets. The minor coat protein pIII interacts with the F pilus of bacteria and is necessary for phage infectivity. The amino terminus of pIII extends into solution so that a protein or peptide fusion can be made without interfering with pIII function. Because of the link between the phage DNA and the protein encoded on its surface, phage display is a powerful screening tool.

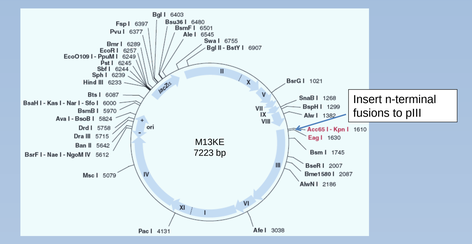


EXPERIMENTAL DESIGN

First, the affibody phage display library was panned against snake venom. We coated a polystyrene plate with a snake venom solution. After incubating the snake venom overnight, unbound venom was washed away. To the coated plate, we added the affibody phage display library. After incubating for 30 minutes, unbound phage were washed from the plate, leaving the phage expressing affibody molecules that bound to the snake venom on the plate. The phage selected during this first round of panning was eluted and amplified. This amplified phage was titered by counting phage plaques, or sites of infection, on a lawn of the α-complementing strain *E.coli* ER2738. The amplified phage from each round was then used in the next round of selection for further amplification. Three rounds of panning were performed. An ELISA assay will be performed on individually selected phage plaques. Affibody phage that are positive on the ELISA will be DNA sequenced to determine the affibody expressed. The sequences of the affibody molecules will be panned against another venom will be analyzed for similarities and differences that might be helpful to determine future directions of the project.



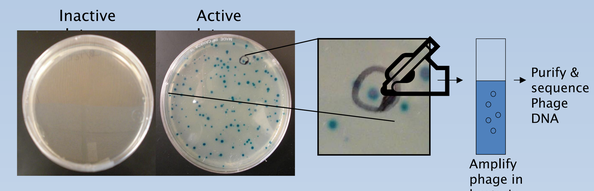
Biolabs, N. E. (n.d.). Ph.D.™-7 Phage Display Peptide Library Kit. Retrieved March 30, 2017, from https://www.neb.com/products/e8100-phd-7-phage-display-peptide-library-kit



Restriction site map of the double stranded plasmid-like of M13KE. The restriction sites Kpn I and Eag I are used to prepare the site for insertion to the n terminus of *gene III*.

PRELIMINARY RESULTS

A direct visual readout of phage activity



As the M13KE is designed to express the lac z alpha domain, blue/white screening provides an immediate readout of phage activity when plated on a x-gal plate with an alpha complementing bacterial lawn. Blue plaques are indicative of M13KE, while white plaques suggest contamination with wild type phage. Phage plaques are then selected and amplified for further identification. Purified phage DNA is used to verify that the DNA has not been mutated. This allows for the identification of the most fit affibody.

Panning Round	Phage Titer (PFU/10 µL)	Amplified Phage Titer (PFU/10 µL)
1	15×10^3	110×10^9
2	17×10^3	100×10^9
3	376×10^3	71×10^9

A series of phage panning was utilized to select the affibody with the highest affinity for the *C. atrox* venom. As the panning proceeds, the affibodies with weak affinities are washed away and those with high affinities are selected for. After 3 rounds of amplification a majority of the G6813A library is still displaying affibody, whereas with unmodified M13KE, most of the clones will have kicked the affibody gene out.

To ensure that the proteases with the venom were not degrading the target proteins before they could be detected, an HPLC trial was run using 1 mg per mL of *C. atrox* venom in Tris pH 6.8 buffer with and without 1mM EDTA. The EDTA acted as a chelating agent to denature the metal containing proteases found within the venom. The HPLC traces were recorded at A 254 and A 260. The traces from each trial were similar across all time periods meaning that there was no detectable protein degradation.

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REFERENCES

1. Newton, J.R. and Deutscher, S. L. 2009. In vivo bacteriophage display for the discovery of novel peptide-based tumor-targeting agents. Methods Mol Biol 504:274-290.
2. Nam, K.T., Lee, Y.J, Krauland, E.M., Kottmann, S.T., and Belcher, A.M. 2008. Peptide-mediated reduction of silver ions on engineered biological scaffolds. ACS Nano 2:1480-1486.
3. Löfblom, J., et al., Affibody molecules: Engineered proteins for therapeutic, diagnostic and biotechnological applications. FEBS Letters, 2010. 584:2670-2680.
4. Calvete, J. J., Fasoli, E., Sanz, L., Boschetti, E., & Righetti, P. G. (2009). Exploring the Venom Proteome of the Western Diamondback Rattlesnake, Crotalus atrox , via Snake Venomics and Combinatorial Peptide Ligand Library Approaches. Journal of Proteome Research, 8(6), 3055-3067. doi:10.1021/pr900249q