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IgY Antibody Production Against Phospholipases A_2 from *Vipera berus* and *Vipera ammodytes* Snake Species

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Introduction

Snakebite envenomation is a neglected disease that has a remarkable impact on Public Health worldwide [1]. In Europe, medically important cases of snakebite are mainly caused by three snake species of the *Vipera* genus (*V. berus, V. aspis*, and *V. ammodytes*). Snake venom is a complex mixture of proteins, peptides, enzymes, etc., and it is known that its toxicity is not a result of all its components [2]. PLA_2s, which are found in the venom of most snakes, are among the most lethal toxins and may represent a promising target to produce a broad-spectrum antivenom [3]. Moreover, currently used snake antivenoms are produced in horses immunized with whole venoms, but the derived antibodies can induce early or late adverse reactions [4]. On the contrary, chickens constitute an alternative to conventional antivenom production due to their economic, productive, and ethical advantages. In this study, two appropriate peptide epitopes from the *C*-terminal segment of PLA_2s of *Vipera berus* and *Vipera ammodytes* snake species were synthesized and conjugated to the CPSOC(3,9Acm) peptide carrier. Hens were used for vaccination with the immunogenic, and the specific IgY antibodies were isolated from the egg yolk to investigate the potency of recognizing both immunogenic conjugates and viper venom.

Methods

Peptide synthesis and Thioether bond formation
The peptide synthesis for both peptides (abbrv: pep1, pep2) was performed by the stepwise solid-phase peptide synthesis on a Rink-Amide (AM) resin using the Fmoc/tBu methodology. Thioether conjugates {abbrv: pep1-CPSOC(3,9Acm), pep2-CPSOC(3,9 Acm)} were formed between the CPSOC(3,9Acm) carrier and the antigenic epitopes. Iodoacetyl-peptides were dissolved in a H_2O/AcN (1:1), carrier CPSOC(3,9Acm) was added to the solution in solid form and small portions. The reaction was performed under inert conditions. At the end of the reaction, the solution was acidified until pH reached 2–3. The peptide and conjugates purity was checked by analytical RP-HPLC, and the correct molecular masses were confirmed by LTQ-ORBITRAP HR-ESI-MS.

Immunization protocol and IgY isolation from egg yolk
Two hens were used for the immunizations. The first one (HenA) was immunized with the pep1-CPSOC(3,9Acm) conjugate and the second one (HenB) with the mixture of both conjugates. For the first immunization, conjugates (0.6mg/ml) were dissolved in 0.5 ml H_2O and mixed with an equal volume of Freund's complete adjuvant, whereas the other three doses consisted of conjugates (0.2 mg/ml) emulsified with Freund's incomplete adjuvant. All doses were administered intramuscularly, in four different places and the eggs were obtained after each immunization. Standard protocol of Polyethylene glycol (PEG) 6000 was used to isolate the IgY antibodies from the egg yolk [5].

Immunochemical assay
To determine the IgY binding activity, ELISA wells were coated with the antigen (5μg/well of conjugates or 1 μg/well of snake venom) at 37°C for 1.5h and blocked with 3% skim milk in PBS for 1h at 37°C. The preimmunization- or fourth-immunization-induced IgY was added to the wells in various dilutions and incubated for 1.5h at 37°C. After removing unbound IgY, the HRP-conjugated anti-chicken IgY was added, and the wells were incubated for another 1h at 37°C. For color development, 3,3′,5,5′tetramethylbenzidine (TMB) substrate solution was added, and the reaction was stopped by adding 0,2N HCl. Absorbance was measured at 450nm.

Results and Discussion

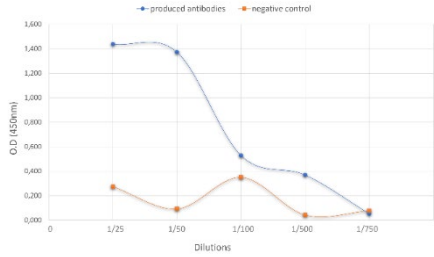

Fig. 1. Schematic representation of the produced antibodies ability to recognize the viper venom.

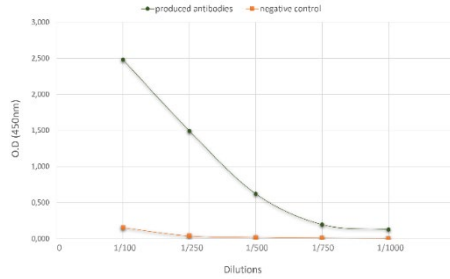

Fig. 2. Schematic representation of the specificity of the produced antibodies against the mixture of the conjugates.

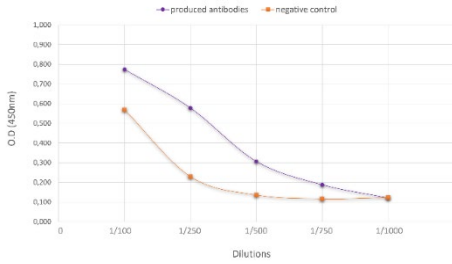

Fig. 3. Schematic representation of the specificity of the produced antibodies against the pep1-CPSOC(3,9Acm) conjugate.

Table 1. Results of the antibody binding activity to pep1-CPSOC(3,9Acm) conjugate (HenA).

Dilutions	Produced antibodies (O.D)	Negative control (O.D)
1/25	1,438	0,274
1/50	1,373	0,092
1/100	0,528	0,350
1/500	0,369	0,042
1/750	0,053	0,076

Table 2. Results of the antibody binding activity to the mixture of immunogenic conjugates (HenB).

Dilutions	Produced antibodies (O.D)	Negative control (O.D)
1/100	2,480	0,152
1/250	1,492	0,039
1/500	0,622	0,018
1/750	0,196	0,009
1/1000	0,128	0,006

Table 3. Results of the antibody binding activity to the viper venom (antibodies from HenB).

Dilutions	Produced antibodies (O.D)	Negative control (O.D)
1/100	0,774	0,469
1/250	0,578	0,229
1/500	0,307	0,136
1/750	0,188	0,116
1/1000	0,121	0,123

The results of this study show that the antibodies produced by the immunization of hens can successfully recognize the immunogenic conjugates. More specifically, the hen immunized with the mixture of conjugates shows a higher antibody titer compared to the one immunized with only pep1-CPSOC(3,9Acm) conjugate. Interestingly, preliminary experiments show strong evidence that the produced antibodies can recognize the *Ammodytes meridionalis* venom, but further research is needed to optimize the assay conditions. Further experiments are in progress to optimize the ELISA assay and examine the ability of antibodies to recognize other viper venoms intending to perform *in vivo* experiments.

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References

1. Scheske, L., et al. *Int. J. Heal. Policy Manag*. **4**(7), 447-457, (2015), https://doi.org/10.15171/ijhpm.2015.75
2. Latinović, Z., et al. *J. Proteomics* **146**, 34-47, (2016), https://doi.org/10.1016/j.jprot.2016.06.020
3. Xiao, H., et al. *Biomed Res. Int*. (2017), https://doi.org/10.1155/2017/6592820
4. León, G., et al. *Toxicon* **76**, 63-76, (2013), https://doi.org/10.1016/j.toxicon.2013.09.010
5. Polson, A., et al. *Immunol. Commun* **9**(5), 475-493, (1980), https://doi.org/10.3109/08820138009066010