Anticoagulant three finger toxin from *Naja siamensis* (Indo Chinese Spitting Cobra)

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ABSTRACT

Snake venoms are gold mines for novel toxins including anticoagulants that can be used to treat thrombotic disorders. We have attempted to isolate an anticoagulant from *Naja siamensis*, an Indochinese black spitting cobra, by employing several separation techniques. Experimental procedures include size exclusion chromatography, reverse phase and cation exchange separation methods. Clotting assays were used to determine the activity and specific site of inhibition in the blood coagulation cascade. Two proteins have been identified as potential anticoagulants: Protein A with molecular mass of 6738.21 ± 1.83 Da and protein B with a molecular mass of 6709.52 ± 1.13 Da. Here, we report that protein A had been purified and it was shown not to be the anticoagulant protein therefore suggesting that protein B is highly likely to be a novel three finger toxin. This is the first time an anticoagulant in the molecular mass range of three finger toxins have been reported in an Asian snake. We have also postulated that protein B is an extrinsic tenase complex inhibitor in the blood coagulation cascade. This novel anticoagulant can be used in designing new drugs with therapeutic use. Efforts to purify protein B are currently ongoing.

INTRODUCTION

Thrombosis has been and continues to be an ongoing problem. In preventing unwanted blood clots, anticoagulant drugs are used to inhibit different stages of the blood coagulation cascade. However due to the inefficiency of these drugs there is a need to look at other sources. One such attractive proposition is snake venom. The venom we chose to work on is from *Naja siamensis* which is found in Southeast Asia. The aim of our project is to find novel three finger toxins with anticoagulant function in *Naja siamensis*.

MATERIALS AND METHODS

Crude venom was size-fractionated using Superdex 30™ gel-filtration column. Two runs of RP-HPLC were carried out at different steps of the purification process. The first run was carried out with Jupiter™ C18 semi prep column to identify the masses of the anticoagulant proteins and the second run was carried out with Jupiter™ C18 analytical column. Two runs of cation exchange

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were then done consecutively. In the first run, cation exchange chromatography was done with UNO™ S6. In the second cation exchange run, the same conditions as the previous run was used but with a different linear gradient. Elution of proteins was monitored at 280 nm and 215 nm UV light. Fraction containing the anticoagulant protein was isolated and lyophilized. Masses of the protein were determined using ESI-MS. Dissection approach (Kini and Banerjee, 2005) was used to determine the specific inhibited step of the blood coagulation pathway by using four different clotting assays: prothrombin time assay, aPTT time assay, thrombin time assay and stypven time assay.

RESULTS

From the size exclusion chromatogram, peaks identified to contain the anticoagulant proteins were pooled together and subjected to RP-HPLC. Two proteins of masses 6738 Da and 6709 Da as referred to as protein A and protein B respectively hereafter were identified as anticoagulant proteins. Cation exchange was done using the pooled fractions from the size exclusion chromatogram and protein A and B were found to be co-eluting in a peak. This peak was subjected to second cation exchange using a shallow gradient. Then, the peak containing protein A was subjected to reverse phase which showed a major peak which contain protein A only. Protein A was lyophilized and the homogeneity was determined by ESI-MS. The molecular mass of the protein purified was 6738.21 ± 1.83 Da (Figure 1). The various clotting assays were done at a concentration of 100 µM of protein A (Figure 2). It can be seen that there were no significant increase in fold activity. Therefore, we have concluded that protein A is not the anticoagulant present. This suggests strongly that protein B could be a novel three finger toxin that has anticoagulant activity. Furthermore, the fold activity is the highest in prothrombin time only and hence Protein B could be a potential extrinsic tenase complex inhibitor.

![Figure 1. Determination of molecular mass and homogeneity of Protein A. Molecular mass was found to be 6738.21 ± 1.83.](image-url)
DISCUSSION

We have reported the discovery of a potential novel three finger toxin with anticoagulant activity which inhibits the extrinsic tenase complex and the evolutionary implication of finding a potential three finger toxin in an Asian snake is discussed.

Protein B is a potential extrinsic tenase complex inhibitor and co-elution of protein A and B

In the clotting assays, the anticoagulant protein prolonged prothrombin time only, but did not prolong Stypven time and thrombin time (Figure 2). This shows that protein B could be a highly specific FVIIa inhibitor that inhibits the extrinsic tenase complex. Synthetic inhibitors have not progressed beyond clinical trials (Shirk and Vlasuk, 2007) and some of them are not highly specific as they are able to inhibit other factors of the blood coagulation cascade (Buckman et al., 2005). FVIIa and TF complex is also reported to induce chemotherapy resistance (Fang et al., 2008). Therefore, protein B could be an interesting proposition for drug design and study aimed that inhibiting FVIIa only. As the efforts to purify protein B are ongoing, a reoccurring challenge is the co-elution of protein A and B in both cation exchange and reverse phase steps. This shows that protein A and B share similar charge and hydrophobic surfaces. They are also likely to be highly similar in their amino acid sequence. The comparison of their sequences would hint at the vital site of the amino acid sequence that inhibits FVIIa.

Potential three finger toxin with anticoagulant activity

Three previous groups (Karlsson and Pongsawasdi, 1980; Betzel et al., 1991; Ohkura et al., 1988) have worked on venom from Naja siamensis and none reported the finding of anticoagulants that were three finger toxins. We have established that in the venom of Naja siamensis, in addition

Figure 2. Clotting assays of Protein A. The four different clotting assays were done using 100µM of protein A. None showed significant increase in fold activity hence indicating that protein A is not an anticoagulant as previously hypothesized.
to protein B being a potential extrinsic tenase complex inhibitor, there is an anticoagulant present which is in the molecular mass range of three finger toxins.

Anticoagulant three finger toxins and cobras

Three finger toxins that have been elucidated so far had been from African cobras. Protein B which is a potential three finger toxin is from an Asian snake. This is revelatory because the divide between Asian and African snakes is deemed to be ancient and probably at the earliest stage of separation (Slowinski et al., 1997; Slowinski and Keogh, 2000). This finding could help us to figure out the mode of evolution for three finger toxins.

CONCLUSION

In summary, Protein A of molecular mass of 6738 Da is not an anticoagulant protein whereas protein B of molecular mass 6709 Da is highly likely to be a novel anticoagulant three finger toxin that specifically inhibits FVIIa in the extrinsic tenase complex. *Naja siamensis* could be the first snake to have an anticoagulant three finger toxin of Asian origin.

REFERENCE


