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Original Research Article

Evaluation of the Coagulant Effect of the Zanjani and Latifi Viper Venom Endemic in Iran

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HIGHLIGHTS

- The viper snake venom is mostly blood-oriented and leads to blood coagulation.
- The snakes venom, including vipers venom endemic of Iran could be applied for pharmaceutical purposes.
- A 26KD protein was the most effective component of viper venom showed plasma coagulation.
- The results showed this protein was related to serine protease enzyme of snake venom.

ABSTRACT

The venom of the viper is very important in pharmaceutical usage, such as in the process of coagulation during medical care. This study aims to evaluate the coagulant factor of Latifi and Zanjani viper venom. In the current study, after electrophoresis of proteins found in viper venom, all of the thick and strong bands of proteins were isolated and prepared for examination of coagulation characteristics, like pro-thrombin time (PTT) and active partial thromboplastic time (APTT), followed by further study by mass spectroscopy. In this way, 11 bands of proteins were recognized in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After PT and APTT tests, one common band in 26 KDa could lead to coagulation of blood plasma in less than one second. Mass spectroscopy identified this band as serine protease isoform4. The results confirmed the coagulation effect of a 26 KDa protein fraction of venom from Latifi and Zanjani vipers.

Introduction

Snakes are peculiar animals as their exact origins remain unknown. Studies on these species focus especially on their venom in order to produce an effective recombinant toxic factor. To achieve the therapeutic goal and design its recombinant type using a biotechnology approach, first, the coagulant factor of viper venom should be recognized (Boyot et al., 1990; Matsui et al., 2000; Clement et al., 2012). Viper venom is mostly blood-oriented. It leads to blood coagulation and, consequently, death of the victim. The main signs of a viper bite are inflammation, local pain along with tissue decay, and organ failure. The venom of a viper causes pain, inflammation, and blushing of skin. Sometimes, it leads to blisters, with plasma and superficial discharges that develop slowly (Kornalík, 1985). Vipers are mostly found on the southern slopes of central Alborz (Rajabizadeh et al., 2012). The venom of this type of viper has a higher lethal factor than other similar types. So, stronger and more effective toxic factors are found in the venom of this type of viper.

Notably, the distance of extinguishment in Iranian viper snake is not endangered. Moreover, it is not unlikely

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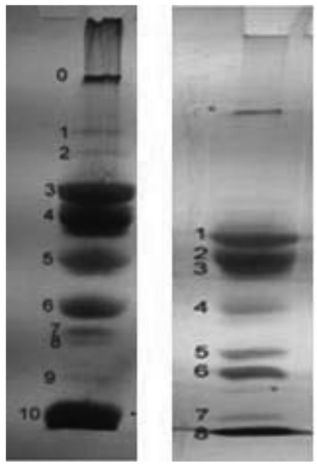

Figure 1. Typical vertical gel electrophoresis of Latifi's viper snake venom (right) and Zanjani's viper snake venom (left).

that indiscriminate harvesting before and after their discovery in 1346 brought them to this stage.

The use of snake venom—including the venom of vipers endemic in Iran—for a variety of pharmaceutical purposes, recently necessitated accurate and scientific research on the pharmacology products derived from these animals, since these creatures are now in danger of extinction. It is necessary to prevent over-hunting of these animals and improve their conservation by breeding them (Kool, 2016; Samy et al., 2016).

The following study was conducted at the Cellular and Molecular Biology Research Center at the Shahid Beheshti University of Medical Sciences. The results confirmed the high quality of Latifi viper venom. To achieve the therapeutic goal and design its recombinant type using the biotechnology approach, first, in order to recognize the coagulant factor of viper venom, we analysed venom proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), prothrombin time (PTT), activated partial thromboplastin time (APTT), Western blotting, and MOLDI-TOF mass spectroscopy.

Material and Methods

Preparing venom of snakes in captivity

The venom samples were obtained from 10 Latifi and Zanjani vipers resident in the laboratory of live animals of the College of Agriculture and Natural Resources at Tehran University. The sampling was performed twice within an interval of one month in the summer. The obtained venoms were transferred to the laboratory of the Cellular and Molecular Biology Research Center at the Shahid Beheshti University of Medical Sciences.

Investigation of coagulation properties of components of snake venom

The venom samples were electrophoresed on 12% acrylamide gel at a voltage of 100V. After three hours, 11 bands from Latifi viper venom and eight bands formed from Zanjani viper venom were separated. Then, each band was homogenized using 200µl sterilized PBS. After 30 minutes, protein purification was carried out to remove the DNA and other debris using a Pierce SDS-PAGE Sample Prep Kit (Thermo Scientific, USA).

Preparing blood plasma samples

The fresh blood samples with 0.11 M sodium citrate (Bahar Afshan, Iran) were collected and centrifuged with 448 g for 10 minutes. The obtained plasma was transferred into clean test tubes.

Coagulation testing of PT assay

To perform the PT test, 50 µl of blood plasma was incubated in a 37°C water bath for three minutes. Then 200µl of leukoplatin and 50µl of purified protein band were added to it. This test was performed separately for each purified protein band.

APTT assay

Since the normal value of PTT was 60–70 seconds, which is a relatively long time, the APTT was also used for purified protein bands. To perform this test, 100µl of leucocephala was added to 50µl of blood plasma. This was then incubated in a 37°C water bath for three minutes. Next, 100µl of calcium chloride (CaCl2) and 50µl of purified protein of viper venom (Latifi and Zanjani) was added to it. This test was performed separately for each band.

MS-MALDI-TOF spectroscopy of effective components of snake venom

The purified protein samples were digested with trypsin and the obtained peptides were studied using MOLDI-TOF or TOF spectrometry. This part of the study was carried out in Proteomics International Pty Ltd Company, Australia. To analyse and identify isolated proteins, Mascot sequence matching software and the Ludwig NR database were used.

Results

SDS-PAGE of Latifi and Zanjani viper venom

As shown in Figure 1, the protein bands of venom obtained from each viper were separated on SDS-PAGE.

Coagulation test of PT of Latifi and Zanjani viper venom

Subsequently, the coagulation effect of bands obtained from the electrophoresis of Latifi viper venom on blood as well as blood plasma was compared by a PT test. The bands 5, 6, and 8 (Fig. 1) obtained from the electrophoresis of Latifi viper venom led to blood clots at the time. In contrast, the blood plasma led to blood clots in these bands within 15 seconds. The reaction was carried out with bands 2, 3, 4, and 9 during 18 to 26 seconds by blood plasma. The coagulation effect of bands obtained from the electrophoresis of Zanjani viper venom on blood as well as blood plasma was compared by a PT test. The bands 4, 5, and 6 obtained from the electrophoresis of Zanjani viper venom led to blood clots at the time. In contrast, the blood plasma formed blood clots with these bands within 14 seconds. The reaction was carried out with bands 1, 2, 3, 7 and 8 during 18 to 26 seconds on blood plasma.

Coagulation test of APTT of Latifi and Zanjani viper venom

The coagulation effect of bands obtained from the electrophoresis of Latifi viper venom on blood as well as blood plasma was also compared using APTT. This test showed results similar to those of the PT test. After determining the bands obtained from the electrophoresis of viper venom that caused rapid blood coagulation, the band 6 (26 KDa) was shown to be the most effective component of plasma coagulation. This band was sent to Proteomics International Pty Ltd Company for analysis using the MOLDI-TOF method. The results of the study showed that this protein was related to the serine protease enzyme of snake venom.

The effective component of snake venom

The sharp protein band in 26 KDa could lead to coagulation of blood plasma in less than one second, as per analysis by MOLDI-TOF mass spectroscopy. The Mascot server showed that the purified protein was serine protease isoform4.

Discussion

The present study sought to evaluate the coagulant factor of viper venom endemic in Iran (Latifi and Zanjani) and as the fact of country's biodiversity values in order to produce the recombinant type and prevent extinction.

The three first bands in Zanjani viper venom gel electrophoresis were weaker than those in Latifi venom. These bands were omitted in further blood coagulation tests and the overall pattern of bands in Zanjani's gel electrophoresis has been considered. Then the sharp and high-quality bands in gel were prepared from both Latifi and Zanjani's gels, and the PT and APTT were carried out on separated protein bands. The results showed that three proteins of SDS-PAGE in two samples of Latifi and Zanjani viper venom have the coagulation effect.

A gel diffusion test was performed on the Zanjani and Latifi viper venom. The study was carried out with 1/10, 1/100, and 1/1,000 poly-valan anti-snake bite antibody dilutions (Razi Institute, Iran), which showed there was substantial antigenic similarity between the two venom samples. The venom of baby vipers had a similar response and the precipitation of the presence of antigen with antisera was observed. But in the second group (Cerastescerastes and Zanjani vipers), there were no acceptable results with the different antibody dilutions (1/10, 1/100, 1/500, and 1/1,000) and snake venom samples.

In addition, in 1391, a master thesis entitled 'Evaluation of the quality of the endemic Latifi viper snake venom (Montivipera latifi)', sought to provide a solution for maintenance in captivity to reduce hunting and enhance protection. This experiment showed that the venom of each species had the same quality and sharp bands. Although the sharpness of Latifi venom was stronger than Zanjani's, the number of bands was equal. In the second step, Latifi viper venom was analysed thrice. Consequently, the results showed the higher quality of this venom (Pourazin et al., 2011).

These two studies formed the basic research on the quality of viper venom. They also provided a comparison between the two. Moreover, the gel diffusion method in the first study evaluated the impact of the venom on antisera in different dilutions.

The current study took one step forward and evaluated the coagulation property of Latifi and Zanjani viper venoms using SDS-PAGE. Following this, the PT and APTT tests were performed with the SDS-PAGE bands formed in two viper venom samples.

In one research study conducted by Rajabizadeh et al. (2012) on the pattern of distribution of Latifi viper snakes with regard to diversity and gender differences, snake venom is quantitatively analysed using dimensional electrophoresis and High Performance Liquid Chromatography (HPLC). Meanwhile, this study was performed with a focus on quality, and the trials were carried out using SDS-PAGE and the Western blotting method.

Toxicology experts in Egypt have studied Cerastescerastes venom to evaluate the detailed specifications in proteins using HPLC and SDS-PAGE (Wahby et al., 2012).

Conclusion

After determining the bands obtained from the

electrophoresis of viper venom, which caused rapid blood coagulation, the 26 KDa protein was identified as the most effective component of plasma coagulation. After analysis by the MOLDI-TOF method, the obtained results revealed that this protein was related to the serine protease enzyme of snake venom.

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