Mem. Inst. Butantan
Simp. Internac.
33(2):519-522, 1966

55. PHARMACOLOGICALLY AND BIOCHEMICALLY ACTIVE COMPONENTS OF JAPANESE OPHIDIAN VENOMS

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We have been making a systematic study on the separation of enzymes of venoms from Japanese and Formosan snakes, and many enzymes were obtained in a pure state.

The most characteristic symptoms produced by injection of the venom of *Agkistrodon halys blomhoffii*, called "Mamushi" in Japanese, a representative of the Japanese poisonous CROTALIDAE snake, are: hemorrhage, necrosis, muscular degeneration and lowering of blood pressure.

Among the enzymes from the venom of *Agkistrodon halys blomhoffii*, one of its three proteinases had hemorrhagic activity, and three were arginine ester hydrolases with "bradykinin releasing", "clotting" and "capillary permeability increasing" activities, they were considered to be connected with the pharmacological actions of the venom. Besides the pharmacologically active enzymes, a striking hemorrhagic protein deprived of any enzymatic activity was also present in the venom. This paper deals with the pharmacologically active components of the venom of *Agkistrodon halys blomhoffii*.

Most of the caseinolytic activities was distributed in three fractions which were designated, in the order of their elution from the column of DEAE-cellulose, as proteinase A, B, and C. Hemorrhagic activity was found mainly in tubes 100 to 170 and 170 to 260. There was little proteolytic activity in the first fraction, designated as HR-I, while in the second fraction, HR-II, there was proteinase B activity. Proteinases A and C were not hemorrhagic factors and no hemorrhage was observed even when they were injected at 300 fold the minimum hemorrhagic dose of proteinase B. When HR-I was injected into a mouse intravenously, marked intestinal hemorrhage was observed, while on injection of HR-II, petechial hemorrhage was observed in the subcutaneous tissues. Next, we attempted to purify HR-II to see whether the hemorrhagic activity was due to the proteinase activity. After HR-II fraction, namely the proteinase B fraction, was desalted by a Sephadex G-25 column, the sample of proteinase B was re-chromatographed on DEAE-cellulose using gradient elution. The curve of absorbancy at 280 mμ did not coincide with the curve of hemorrhagic or proteinase activity, these latters being found in a single peak. After lyophilization, the sample was applied to a hydroxylapatite column, and the hemorrhagic activity was eluted together with the caseinolytic activity. To purify the resulting proteinase B preparation, DEAE-Sephadex A-25 column chromatography was used. By this procedure some impurities were removed. The hemorrhagic and caseinolytic activities of proteinase B were not separated by these purification procedures and

the increase in potency of the homorrhagic activity at each step was essentially in parallel with that of the caseinolytic activity of proteinase B. The average yields of the purified proteinase B, from crude venom was about 2.5 per cent. The purified preparation was chromatographically, electrophoretically and also ultracentrifugally homogeneous.

Proteinase B showed to be a metal-protein, and the hemorrhagic and caseinolytic activities fully remained even after the removal of sialic acid by sialidase. But its activities were inhibited by EDTA and cysteine. In EDTA-inhibition experiments, the extent of the decrease in hemorrhagic activity was parallel with the decrease in caseinolytic activity. Also, in cysteine-inhibition experiments, the hemorrhagic activity decreased parallel with the caseinolytic activity, in proportion to the amount of cysteine added.

There was another hemorrhagic fraction, namely the fraction of HR-I containing arginine esterases, from which the arginine esterases were easily removed by hydroxylapatite chromatography. The HR-I preparation thus obtained contain no enzyme activity. Although the protein content of this HR-I preparation was low, the toxic principle must be a protein and when this was treated with various proteinases, the hemorrhagic activity decreased.

As the hemorrhagic activity of HR-I appeared to play a leading role in the lethality of the venom of *Agkistrodon halys blomhoffii*, a comparison of the hemorrhagic and proteinase activities in various venoms were examined. The crude venom of *A. contortrix contortrix* had no hemorrhagic activity even though it had high proteinase activity. On the other hand, the venom of *Crotalus adamanteus*, which showed strong hemorrhagic activity had low proteinase activity. Moreover, when the latter venom was fractioned on a DEAE-cellulose column, 98 per cent of the total hemorrhagic activity was recovered in a fraction which had no proteinase activity. This hemorrhagic factor was pharmacologically different from HR-I isolated from *Agkistrodon halys blomhoffii* venom. So, it seems that even in venoms of the same family, there may be different hemorrhagic factors and different substances with lethal toxicity, and for the characterization of these toxic principles, it is essential to purify each factor from each snake venom.

In the same way, we purified two kinds of arginine esterases, namely the "clotting" and the "capillary permeability increasing enzymes" in physico-chemically pure states. We also obtained the bradykinin releasing enzyme, free from other physiologically active components.

As a considerable amount of the "clotting enzyme" and the "capillary permeability increasing enzyme" were present in the venom, we attempted to purify the first to a physico-chemically homogeneous state. Purification procedures consisted of four steps and by these procedures the clotting enzyme was purified to a physico-chemically homogeneous state.

Among the arginine ester hydrolases in the eluate from the DEAE-cellulose column, an enzyme which has a hypotensive action and increases capillary permeability was found. The arginine ester hydrolytic activity of this enzyme was 30 to 40 per cent of the total arginine ester hydrolytic activity of the venom, and when it was injected into the skin of an albino rabbit, the permeability of the capillaries were distinctly increased as shown by the Evans Blue Test.

When this preparation was incubated with purified bradykininogen, no release of bradykinin was detected by assay on guinea-pig ileum. So, it is not clear by what mechanism the permeability of the capillaries is increased by this enzyme.

In other experiments, we found that a considerable amount of this capillary permeability increasing enzyme was also present in *Crotalus adamanteus* venom and in *Trimeresurus flavoviridis* venom. This enzyme of the venom of *Agkistrodon halys blomhoffii* was purified by similar procedures as those which were used for the clotting enzyme. The specific activity of this enzyme was more than 70-fold that of the crude venom, and the purified preparation was homogeneous on ultracentrifugation and cyanogum electrophoresis at various pH values.

The ultracentrifugation patterns of the "clotting" and "capillary permeability increasing" enzymes show the homogeneities of the enzymes.

When the purified "clotting enzyme" was incubated with 98 per cent pure fibrinogen, which had been prepared in our laboratory, according to the method of Blombäck and Laki, three kinds of fibrinopeptides were liberated. Two of these corresponded to fibrinopeptides A and B which were liberated from fibrinogen by the action of thrombin or by the venom of *Bothrops jararaca*. But, one of the three peptides was a new fibrinopeptide. By amino acid analysis and end group analysis of this new fibrinopeptide, it was concluded to be a peptide which resulted from the loss of one mole of arginine from the C-terminus of fibrinopeptide B. The reason why the physico-chemically pure "clotting enzyme" of the venom, which showed only arginine ester hydrolytic activity, liberated this new peptide is not yet known. When the enzyme was incubated with fibrinopeptide B, no reaction was observed.Therefore, the new fibrinopeptide seemed to be released directly from the bovine fibrinogen molecule by the action of the clotting enzyme.

The clotting activity of the enzyme of *Agkistrodon halys blomhoffii* was lower than that of the clotting enzyme of *Bothrops jararaca*. The activity of the clotting enzyme of *Agkistrodon halys blomhoffii* was not inhibited by plasma anti-thrombin. In this it is similar to the clotting enzyme of th evenom of *Bothrops jararaca*.

Bradykinin releasing enzyme fraction which was obtained from the eluate on the first DEAE-cellulose column chromatography of *Agkistrodon halys blomhoffii* venom, contained clotting enzyme. And it was further applied to a CM-cellulose column. Thus the bradykinin releasing enzyme, free from clotting enzyme, was obtained, but only 5 per cent of the total units of arginine ester hydrolytic activity of the venom were recovered in this partially purified enzyme preparation. Therefore, no further purification was attempted. Although the substrate specificities of the clotting, bradykinin releasing, and capillary permeability increasing enzymes were qualitatively the same, their physiological activities were completely different. The bradykinin releasing was only inhibited by trasylol, which is a potent inhibitor of urinary and pancreatic kallicreins, and the clotting and capillary permeability increasing enzymes were not inhibited by trasylol at all. From these results and the specificities found by tests on several synthetic substrates, the bradykinin releasing enzyme in the venom seems to be a salivary kallikrein of the snake.