PROTEOLYTIC ACTIVITIES OF HABU SNAKE VENOM AND THEIR SEPARATION FROM LETHAL TOXICITY

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It is generally recognized that most of snake venoms manifest proteolytic activity (Zeller, 1951). Several reports have recently been accumulated (Deutsch et al., 1955; Rao et al., 1956; Hadidian, 1956; Henriques et al., 1958; Maeno et al., 1958) to suggest the presence of more than one proteolytic enzymes in certain snake venoms.

Up to date, comparatively numerous informations have been available on the pharmacological function of the potent proteolytic enzymes of the Crotalidae venoms. Hemorrhage and necrosis are claimed to be attributable, at least in part, to the action of proteolytic enzymes (Houssay, 1930; Kellaway, 1939; Zeller, 1948; Porges, 1953; van Heyningen, 1954; Slotta, 1955; Kaiser et al., 1958; Maeno et al., 1958). In our recent studies (Ohsaka et al., 1960, 1961) it was reported that zone electrophoretical fractionation, coupled by a new quantitative method for estimating hemorrhagic activity (Kondo et al., 1960), of Habu venom revealed the presence of at least two hemorrhagic principles, both of which were associated with proteolytic activity on casein. Eagle (1937) and Jánszky (1950) demonstrated a parallelism between the proteolytic activity of venoms and their blood coagulating activity. The proteolytic enzymes may also contribute to the hypotensive action of snake venom, resulting in shock, through damage given to vascular endothelium with consequent escape of blood from circulation (Kellaway, 1939; Porges, 1953) or through the liberation of bradykinin from serum globulin (Rocha e Silva et al., 1949; Prado et al., 1950).

In view of the pharmacological function of proteolytic enzymes of Crotalidae venoms above-cited, and of the fact that such venoms also manifest lethal toxicity, it becomes of obvious interest and significance to know whether or not proteolytic activity in Habu snake venom can contribute to its lethal toxicity.

The experiments here reported indicate that at least the main part of lethal toxicity and of proteolytic activity on casein were independent of each other. Evidences suggesting the presence of several proteolytic enzymes in this venom will also be presented.

MATERIALS AND METHODS

Snake venom: The venom used was a dried and powdered pool (Batch No. 48) from a species of Habu, Trimeresurus flavoviridis (Hallowell).

Substrates: Purified casein was purchased from the Merck and Co., Inc., Rahway, N. J., U. S. A. The 'azocoll' (Oakley et al., 1946) and collagen (Noda, 1955) were generously supplied by Dr. H. Nogak of the University of Tokyo.
Experimental animals: An inbred strain of white mice (strain: general purpose colony, Yoken) of both sexes weighing from 14 to 17 g was used to assay lethal toxicity.

Estimation of protein: The protein content of a venom solution was estimated in terms of its ultraviolet absorption at 280 m\(\mu\) in the 1 cm cell of a Beckman DU spectrophotometer.

Estimation of proteolytic activity: The proteolytic activity was determined with casein, 'azocoll' or native collagen as substrate. When casein was used as substrate a modified method of Kunitz (1946) was applied. To the mixture of 1.0 cc of enzyme solution and 0.5 cc of 0.2 M phosphate buffer of pH 7.5, was added 0.5 cc of 4\% (w/w) casein dissolved in 0.1 M disodium phosphate solution. After 10 minutes of incubation at 35\(^\circ\)C, the reaction was stopped by the addition of 2.0 cc of 0.4 M trichloroacetic acid. The excess casein precipitated was removed by filtration. The extent of digestion was evaluated by determination of the absorption at 280 m\(\mu\) of the trichloroacetic acid filtrate with a Beckman DU spectrophotometer. For the assay of effects of metal ions and EDTA (disodium ethylenediamine tetraacetate) on the enzyme activity the following system was used: A mixture of 0.5 cc of 4\% (w/w) casein dissolved in 0.05 M veronal-HCl buffer (pH 7.5), 0.9 cc of the same buffer (pH 7.5) and 0.5 cc of enzyme solution was added with 0.1 cc of the above substances given in a concentration of 2 \(\times\) 10\(^{-2}\) M. The relation between enzyme concentrations and enzyme activity using veronal-HCl buffer system is shown in Fig. 1.

The proteolytic activity on 'azocoll' (Oakley et al., 1946) was determined by a modified method of Ridwell and van Heyningen (1948). Two-tenths cc of the enzyme solution was added to 5.0 cc of the azocoll suspension (4 mg azocoll/cc of 0.05 M borax-boric acid buffer, pH 7.4). In cases where effects of metal ions and EDTA on enzyme activity were to be determined, 0.1 cc of these substances in a concentration of 2 \(\times\) 10\(^{-2}\) M was added to the above mixture. The mixture was incubated for one hour at 37\(^\circ\)C with occasional shakings. The reaction was stopped by filtering off the remaining substrate through a filter paper. The intensity of color of the resulting filtrate was measured at 520 m\(\mu\) with a Coleman spectrophotometer. The relation between enzyme concentrations and enzyme activity is shown in Fig. 2.

The proteolytic activity on native collagen (Noda, 1955) was tested by a modification of Neuman and Tytell (1950). One cc of 0.2\% collagen dissolved in 0.2\% acetic acid, 1 cc of 1/60 N NaOH and 2.0 cc of 0.05 M borax-boric acid buffer of pH 7.4 were mixed in each tube to give the sus-
pension of fibrous collagen. To this were added 0.1 cc of serial 1.5-fold dilutions of an enzyme sample and reaction started. After incubation for one hour at 37°C, activity was expressed as the highest dilution of the enzyme solution enough to digest fibrous collagen. However, no activity was detected in this venom even though 0.5 to 2.0 mg of crude venom was incubated for several hours at 37°C. This result was in good accordance with Kaiser's finding (Kaiser 1958).

Assay of lethal toxicity: As reported in the separate paper (Ohsaka et al., 1961), lethal toxicity was assayed by intravenous injections of white mice with four to five doses of each fraction graded with 1.4-fold intervals in the amount of 0.2 to 0.5 cc. Five animals were injected with each dose. The LD₅₀ (50 per cent lethal dose) was calculated by the Reed-Muench method. The standard error of the LD₅₀ was calculated according to Pizzi (1950). The fiducial limits of the LD₅₀ were about 30% at 5% risk (Ohsaka et al., 1961). The LD₅₀ of the crude venom used (Batch No. 48) was 61 µg ± 3 µg for mice weighing from 15 to 17 mg.

Zone electrophoresis: Zone electrophoresis using starch as a supporting medium was carried out in two types of apparatus. Commercial potato starch was thoroughly washed and treated with the buffer as previously described (Ohsaka, 1958). All runs were made in borax-NaOH buffer of pH 9.2–9.3 and 0.1 ionic strength in a cold room (2–4°C). The trough experiments were performed in an apparatus similar to that of Kunkel and Slater (1952). In an experiment shown in Fig. 4, the sample, amounting about 510 mg of crude venom in 5.1 cc of physiological saline was introduced into a 1 cm slit cut crosswise in a 50 × 5 × 1.5 cm from the anodal end. A current of 18 mA was maintained for 24 hours by a potential drop of about 225 volts. After the electrophoresis was finished, the starch block was cut into 1 cm segments, each of which was eluted with 15 cc of physiological saline. Estimation of protein, lethal toxicity and proteolytic activity on casein were made on appropriate aliquots of each eluate.

The column experiment described in this paper (Fig. 3) was the same electrophoretic run, part of whose results was reported in a separate report (Ohsaka et al., 1961). Two grams of Habu venom were subjected to electrophoresis in a column of 3.6 × 114.5 cm packed with starch. A current of 20 mA with a potential difference of 380 volts was applied for about 75 hours. After the electrophoresis ended the starch column was eluted with the same buffer and 10.6 cc fractions were collected. Amount of protein, proteolytic activities on casein and on azocoll, and lethal toxicity were determined.

RESULTS

Comparison of Some Properties of Electrophoretic Components of Proteolytic Activity

The elution diagram, obtained when Habu venom was submitted to zone electrophoresis in a packed starch column (borax-NaOH, pH 9.26, μ=0.1), is presented in Fig. 3. The optical density at 280 mμ for protein, lethal toxicity, and proteolytic activity on casein and on azocoll are plotted for each fraction. Ninety-seven per cent for protein, 71 per cent for lethal toxicity, 100 per cent for proteolytic activity on casein and 53 per cent for that on azocoll were recovered.

As can be seen in Fig. 3, this venom contained at least five electrophoretic components with proteolytic activity on casein. The possibility that the electrophoretic components represent distinct enzymes was then investigated.

In Table 1, the actions on casein and azocoll of five electrophoretic components of proteolytic activity are compared. Specific activity and activity ratio with the two substrates of each fraction are also shown. It can be seen from this Table that fraction No. 22 had a rather weak action on azocoll as compared with fractions No. 14, No. 17 or No. 20 and that fraction No. 24 had no action on azocoll. Therefore, two of the proteolytic fractions (No. 22 and No. 24) could be differentiated from the others by preference towards substrates.

Effects of metal ions and EDTA (final concentration 10⁻³ M) upon proteolytic activities on casein and on azocoll are summarized in Table 2. All venom fractions as well as the
The activity of crude venom was activated by Ca ion, whose effect was also confirmed when EDTA-dialyzed crude venom was used (95% activation). It was also noted that Co and Cd ions were strong inhibitors for the activity of crude venom (63% and 100% inhibition, respectively).

So far as the venom fractions are concerned, activities both on casein and on azocoll of all fractions were greatly inhibited by EDTA and activities on casein of fractions No.

Table 1. Comparison of hydrolytic action on casein and azocoll of various fractions separated by starch column electrophoresis

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>% Recovery of activity</th>
<th>Specific activity</th>
<th>Activity ratio (C/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Casein as substrate (C)</td>
<td>Azocoll as substrate (A)</td>
<td></td>
</tr>
<tr>
<td>Crude venom</td>
<td>100%</td>
<td>100%</td>
<td>1.0</td>
</tr>
<tr>
<td>Fraction No. 14</td>
<td>10.9</td>
<td>5.4</td>
<td>2.0</td>
</tr>
<tr>
<td>17</td>
<td>12.5</td>
<td>7.7</td>
<td>1.6</td>
</tr>
<tr>
<td>20</td>
<td>7.7</td>
<td>3.8</td>
<td>2.0</td>
</tr>
<tr>
<td>22</td>
<td>5.3</td>
<td>0.85</td>
<td>6.2</td>
</tr>
<tr>
<td>24</td>
<td>2.1</td>
<td>0</td>
<td>∞</td>
</tr>
<tr>
<td>10—25</td>
<td>99.9</td>
<td>53.1</td>
<td></td>
</tr>
</tbody>
</table>

Electrophoretic run, as well as fraction No., is the same as described in Fig. 3. Specific activities of proteolytic activities are expressed as the ratio to those of the crude venom. All the reactions were performed under the conditions described in the text.
14 and No. 17 were inhibited to some extent by Mn and Ca ions while that of fraction No. 20 was inhibited by Mn ion but activated to some extent by Ca ion. Therefore, the proteolytic fraction No. 20 could be differentiated from the fractions No. 14 and No. 17.

In view of the above results these electrophoretic components could be eventually categorized into at least three distinct enzymes, i.e., the azocoll-disintegrating group which can not be activated by Ca ion (No. 14, No. 17), the azocoll-disintegrating group which can be activated by Ca ion (No. 20) and the azocoll non-disintegrating group (No. 22, No. 24).

Table 2. Effects of metal ions and EDTA upon proteolytic activities on casein and on azocoll of several fractions separated by starch column electrophoresis

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Casein as substrate</th>
<th>Azocoll as substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>Mn</td>
</tr>
<tr>
<td>Crude venom</td>
<td>100</td>
<td>94</td>
</tr>
<tr>
<td>Fraction No. 14</td>
<td>100</td>
<td>82</td>
</tr>
<tr>
<td>17</td>
<td>100</td>
<td>72</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>&lt;55</td>
</tr>
</tbody>
</table>

Electrophoretic run, as well as fraction No., in this Table is the same as in Fig. 3. All fractions were previously dialyzed against 0.85% NaCl for about 24 hours at 2-4°C. Solution of metal ion or EDTA (final concn. $10^{-3}$ M) was incubated for 15 minutes at 35°C with the venom before addition of the substrate.

**Relations of Lethal Toxicity to Proteolytic Activity on Casein and on Azocoll**

As seen in Fig. 3, proteolytic activity peaks obtained in fractions No. 14 and No. 17 by the use of casein can be separated from the main part of lethal toxicity (fractions No. 17-26). It is also indicated that proteolytic activity peaks in fractions No. 13, No. 15 and No. 17 obtained with azocoll can also be separated from the main part of lethal toxicity.

In order to separate more sufficiently proteolytic activity on casein from lethal toxicity, zone electrophoresis of a rather small amount of *Habu* venom in starch block was carried out under the same conditions as the foregoing experiment. Experiments of this design were carried out more than five times, consistently showing identical patterns. Results are shown in Fig. 4.

Recovery was 104 per cent for protein, 101 per cent for proteolytic activity on casein and 66 per cent for lethal toxicity. Essentially the same pattern as seen in Fig. 3 was obtained, and the resolution was much better than that in Fig. 3. It was again recognized that at least five peaks of proteolytic activity on casein were present. Specific activities of proteolytic activity on casein and of lethal toxicity of the fractions are recorded in Table 3. The total recovery of proteolytic activity in four peaks (No. -21 to No. -9) was 94 per cent, while that of lethal toxicity present in these fractions was 14 per cent. On the other hand, 7 per cent of proteolytic activity on casein was involved in the main lethal toxicity peak (52% covering fractions No. -8 to No. -2).
Table 3. Specific activities of proteolytic activity on casein and of lethal toxicity of fractions separated by zone electrophoresis in starch block

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>Specific activity of proteolytic activity on casein</th>
<th>Specific activity of lethal toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude venom</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Fraction No.</td>
<td>5.5</td>
<td>0.4</td>
</tr>
<tr>
<td>-17</td>
<td>6.9</td>
<td>0.7</td>
</tr>
<tr>
<td>-15</td>
<td>6.4</td>
<td>0.4</td>
</tr>
<tr>
<td>-14</td>
<td>5.4</td>
<td>0.8</td>
</tr>
<tr>
<td>-12</td>
<td>4.6</td>
<td>0.8</td>
</tr>
<tr>
<td>-10</td>
<td>0.30</td>
<td>1.0</td>
</tr>
<tr>
<td>-5</td>
<td>0.04</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Electrophoretic run, as well as fraction No., in this Table is the same as described in Fig. 4. Specific activity is expressed as the ratio to those of the crude venom.

In view of the above results it is indicated that the main parts of lethal toxicity and of proteolytic activity on casein little corresponded to each other.
Recently, several authors (Deutsch et al., 1955; Rao et al., 1956; Hadidian, 1956; Henriques et al., 1958) suggested the presence of more than one proteolytic enzymes in snake venoms. Maeno and Mitsuhashi (1958) also worked with the venom of Habu and suggested the presence of two proteolytic enzymes as observed with casein as substrate, which could be fractionated by solubility in ammonium sulfate solution. In the present study, it was recognized that Habu venom contained at least five electrophoretic components with proteolytic activity on casein (Figs. 3 and 4). Evidences were obtained to suggest that two of the components (fractions No. 22 and No. 24) were different from the others in respect to preference towards substrates (Table 1) and that one of them (fraction No. 20) was different from the others in respect to activation by a metal ion (Table 2). We found Ca ion activation of proteolytic activity on casein (Table 2) in good accordance with Henriques et al.'s finding (Henriques et al., 1958). These components, therefore, could be categorized eventually into at least three distinct enzymes.

Proteolytic enzymes of the Crotalidae venoms have been known to have several pharmacological functions. Hemorrhage and necrosis are believed to be due, at least in part, to the action of proteolytic enzymes (Houssay, 1930; Kellaway, 1939; Zeller, 1948; Porges, 1953; van Heyningen, 1954; Slotta, 1955; Kaiser et al., 1958; Maeno et al., 1958). In our recent studies (Ohsaka et al., 1960, 1961) a quantitative analysis of electrophoretic fractions demonstrated that Habu venom contained at least two hemorrhagic principles, designated as HR1 and HR2, both of which were associated with proteolytic activity on casein. Attempts to separate HR1 and lethal toxicity into separate fractions were unsuccessful (Ohsaka et al., 1961). The proteolytic enzymes may also contribute to the hypotensive action of snake venom through damage given to vascular endothelium with consequent escape of blood from the vessels (Kellaway, 1939; Porges, 1953) or through the liberation of bradykinin from serum globulin (Rocha e Silva et al., 1949; Prado et al., 1950). This action might produce shock phenomena.

Considering the above informations, together with the fact that proteolytic enzymes have high toxicity (Kellner et al., 1954), present experiments were directed to elucidate whether or not proteolytic activity in Habu snake venom can cause its lethal toxicity. The data presented herein indicate that the main part (52% comprising fractions No. -8 to No. -2) of lethal toxicity can be separated from almost all parts (94%) of proteolytic activity on casein (Fig. 4). Proteolytic activity on casein involved in the main lethal toxicity peak was only 7 per cent. Of course, we can not rule out the possibility that this minor part of proteolytic activity might be responsible for such a large part of lethal toxicity. Specific activities of proteolytic activity on casein were easily increased by 4-7 fold by means of one zone electrophoretic run in a starch block (Table 3), suggesting that such a fractionation might be useful for purification of these enzymes.

**SUMMARY**

Zone electrophoretic fractionation of Habu venom revealed that at least five electrophoretic components of proteolytic activity on casein were present in this venom and could be categorized into at least three distinct enzymes.

It was also indicated that at least the main part of lethal toxicity and that of proteolytic activity on casein were independent of each other.
The author is very grateful to Dr. D. Mizuno for his constant encouragement and advices throughout this work. He also wishes to express his thanks to Dr. H. Ikezawa, Dr. H. Kondo and Dr. S. Kondo, of the Department of Serology, for their useful collaboration.

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HABU SNAKE VENOM

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