Isolation and characterization of the thrombin-like enzyme from Cryptelytrops purpureomaculatus venom

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A B S T R A C T

A thrombin-like enzyme, purpurase, was purified from the Cryptelytrops purpureomaculatus (mangrove pit viper) venom using high performance ion-exchange and gel filtration chromatography. The purified sample (termed purpurase) yielded a homogeneous band in SDS-polyacrylamide gel electrophoresis with a molecular weight of 35,000. The N-terminal sequence of purpurase was determined to be VVGGDECNINDHRSLVRIF and is homologous to many other venom thrombin-like enzymes. Purpurase exhibits both arginine ester hydrolysis and amidase activities. Kinetic studies using tripeptide chromogenic anilide substrates showed that purpurase is not fastidious towards its substrate. The clotting times of fibrinogen by purpurase were concentration dependent, with optimum clotting activity at 3 mg fibrinogen/mL. The clotting activity by purpurase was in the following decreasing order: cat fibrinogen > human fibrinogen > dog fibrinogen > goat fibrinogen > rabbit fibrinogen. Reversed-phase HPLC analysis of the products of action of purpurase on bovine fibrinogen showed that only fibrinopeptide A was released. Indirect ELISA studies showed that anti-purpurase cross-reacted strongly with venoms of most crotalid venoms, indicating the snake venom thrombin-like enzymes generally possess similar epitopes. In the more specific double-sandwich ELISA, however, anti-purpurase cross-reacted only with venoms of certain species of the Trimeresurus complex, and the results support the recent proposed taxonomy changes concerning the Trimeresurus complex.

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1. Introduction

Pit viper venoms are rich sources of thrombin-like enzymes (Castro and Rodrigues, 2006; Pirkle and Stocker, 1991). These enzymes have been widely investigated because of their clinical importance (Stocker, 1990, Phillips et al., 2009). The enzymes typically hydrolyze fibrinogen to yield soft fibrin clots which are easily removed from the circulation. Many thrombin-like enzymes have been purified from snake venoms (for example, Koh et al, 2001; Magalhaes et al., 2007; Sakai et al, 2006; Wei et al., 2007) and several have been cloned (Pan et al., 1999; Yang et al., 2005; Zha et al., 2006). At present, more than 30 snake venom thrombin-like enzyme amino acid sequences are known, and they share the same active site sequence motif (Castro et al., 2004). Since there are differences among these venom enzymes in their specific thrombin-like actions, their comparative study offers an attractive approach to defining the structural basis for their coagulant action as well as advancing our understanding of their potential therapeutic applications. Cryptelytrops purpureomaculatus (mangrove pit viper, formerly known as Trimeresurus purpureomaculatus) is a medically important poisonous land snake in Malaysia (Tan, 1991). The venom of C. purpureomaculatus exhibits moderate procoagulant activity (Tan and Tan, 1988). In this paper, we present the results of biochemical and immunological studies on the major thrombin-like enzyme of the venom.

2. Materials and methods

2.1. Materials

C. purpureomaculatus and Calloselasma rhodostoma (Malayan pit viper) venoms were obtained from Latoxan (Valence, France). Sephadex G-25, Resource Q strong anion exchange column (6 mL) and Superose 6 HR 10/2 column were obtained from GE Healthcare (UK). Supercoils LC-18 reversed-phase column (250 × 4.6 mm, pore size 100 Å) was obtained from Supelco (Belfonte, PA, USA). MA7Q anion exchange column (50 × 7.8 mm) was obtained from Bio-Rad Laboratories (Richmond, CA, USA). All other reagents, plasma, enzymes and enzyme substrates were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Isolation and purification of the thrombin-like enzyme from C. purpureomaculatus venom and C. rhodostoma venom

C. purpureomaculatus venom (100 mg) was fractionated by Resource Q strong anion exchange column and eluted by a linear gradient of 16–26% (0.5 M NaCl in 20 mM Tris–HCl, pH 8.0) with a flow rate of 2 mL/min. The thrombin-like enzyme fractions were pooled, desalted and concentrated by freeze-drying. The sample was redissolved in 20 mM Tris–HCl, pH 8.0...
and further purified by Superose 6 column. Ancrod, the thrombin-like enzyme from C. rhodostoma venom was purified by a combination of Resource Q ion exchange chromatography followed by MA7Q anion exchange chromatography.

2.3. General methods

Protein concentration was determined according to Bradford (1976). SDS-polyacrylamide gel electrophoresis was conducted according to Studier (1973). Molecular weight of the purified sample was determined using Superose 6 column with albumin, ovalbumin, carbonic anhydrase, chymotrypsinogen A and ribonuclease A as molecular weight markers. Arginine esterase activity was determined according to Collins and Jones (1972) with α-benzoyl arginine ethyl ester (BAEE) as substrate. Amidase activity was determined using the method described by Svendsen and Stocker (1977).

2.4. Fibrinogen clotting activity

Fibrinogen clotting time was determined by mixing 20 µL (1 µg) of enzyme with 200 µL of bovine fibrinogen (1.5 mg/mL in 0.15 M NaCl) equilibrated at 37 °C. The clotting time of fibrinogen from different animal sources was determined by mixing 200 µL of the various fibrinogens (3 mg/mL in 0.15 M NaCl) and 1 µg of thrombin-like enzyme, at 37 °C.

2.5. Fibrinogenolytic activity

Fibrinogenolytic activity was examined using 1% bovine fibrinogen in 0.15 M NaCl. The enzyme (10 µg) was added to the solution, followed by incubation at 37 °C. Aliquots of the reaction mixture were withdrawn at various time intervals and denatured by boiling for 5 min, and then the clots that formed were removed by centrifugation at 8000 g for 10 min. The supernatant containing fibrinopeptides was analyzed by reversed-phase HPLC. The solution (100 µL) was loaded on reversed-phase C18 column (0.4 cm×22 cm) and then analyzed essentially as described by Shimokawa and Takahashi (1995).

2.6. N-terminal sequencing

N-terminal sequencing was carried out by automatic Edman degradation using pulsed liquid type Sequenator (Applied Biosystems, Model 477A) equipped with an online PTH analyzer.

2.7. Production of antibodies against the purified thrombin-like enzyme

Rabbits were injected intramuscularly with the antigen (purified thrombin-like enzyme). For the first two injections, the venom/venom component (10 µg) dissolved in PBS was mixed with an equal volume of Freund’s complete adjuvant and the mixture was injected intramuscularly into the left and right thighs of the rabbits alternatively at 0th and 3rd week. For subsequent booster immunization, the venom/venom component (20 µg) was dissolved in 0.5 mL of PBS (pH 7.2) and injected intramuscularly at multiple sites at the back of the rabbit on the 6th and 9th week. The rabbits were anaesthetized and bled by heart puncture 9 days after the final injection.

2.8. Purification of IgG and preparation of the horseradish peroxidase (HRP) conjugate

IgG antibodies were purified using protein A affinity chromatography and the IgG-HRP conjugate was prepared as described by Tijssen (1985).

2.9. Indirect and double-sandwich ELISA

Indirect and double-sandwich ELISA were carried out as reported by Harman and Pawlik (1982), as modified in Tan et al. (1993). For indirect ELISA, microtiter plates were coated overnight at 4 °C with 100 µL of venom (20 ng/mL). Plates were then incubated with 100 µL of IgG anti-purpurase (1:500) for 1 h at room temperature, followed by incubation of 100 µL goat anti-rabbit IgG-HRP conjugate (1:4000) for 1 h. Substrate (100 µL; 0.4 mg/mL o-phenylenediamine dihydrochloride and 0.003 % H2O2 in 0.1 M citrate-phosphate buffer, pH 5.0) was then added and the enzymatic reaction was terminated 10 min later with the addition of 50 µL of sulfuric acid (12.5 %). Absorbance at 492 nm was then measured. For double-sandwich ELISA, the microplates were coated overnight at 4 °C with 100 µL of the IgG anti-purpurase (6 µg/mL). Plates were then incubated with 100 µL of venom (300 ng/mL) for 2 h at room temperature. This was followed by incubation of 100 µL of IgG anti-purpurase-HRP conjugate (1:500) for 2 h. 100 µL of substrate was then added, and the enzymatic reaction was terminated 10 min later with the addition of 50 µL of sulfuric acid (12.5%). Absorbance at 492 nm was then measured.

3. Results

3.1. Purification of the thrombin-like enzyme from C. purpureomaculatus venom

Resource Q ion exchange chromatography of the crude C. purpureomaculatus venom yielded 9 peaks. Thrombin-like activity occurred mainly in peak 6 (Fig. 1). Superose 6 gel filtration chromatography of peak 6 yielded 6 peaks (Fig. 2). Thrombin-like activity occurred mainly in peak 1. Peak 1 is the purified thrombin-like enzyme and is termed purpurase.

3.2. Physical characterization of purpurase

Purpurase exhibited a single, homogeneous band in SDS-PAGE with a molecular weight of 35,000. The molecular weight of purpurase

![Fig. 1. Resource Q anion exchange chromatography of C. purpureomaculatus venom. The venom (100 mg) was applied to a prepacked Resource Q column preequilibrated with 20 mM Tris–HCl, pH 8.0. The column was eluted by a linear gradient of 16–26% (0.5 M NaCl in 20 mM Tris–HCl buffer, pH 8.0) with a flow rate of 2 mL/min. Fraction volume was 1 mL. Peak 6 exhibited thrombin-like enzyme activity.](image-url)
as determined by Superoxide gel filtration chromatography was 38,750. The N-terminal sequence of purpurase was determined to be VVGGDECNIN DHRSLVRIF.

3.3. Kinetic studies

The \( K_m \) and \( k_{cat} \) values for purpurase with BAEE as substrate were 0.09 mM and 3.58 s\(^{-1}\), respectively. The kinetic parameters of the action of purpurase on tripeptide anilide substrates are shown in Table 1. N-p-tosyl-glycine-L-proline-L-lysine was the best substrate with the highest value of \( k_{cat}/K_m \).

3.4. Effects of fibrinogen concentration on the clotting time of purpurase

Table 2 shows that the fibrinogen clotting time of purpurase was dependent upon the concentration of the fibrinogen, with the shortest clotting time when fibrinogen concentration was 3.0 mg/mL. The clotting time prolonged substantially when fibrinogen concentration exceeds 5.0 mg/mL. For comparison purpose, the effects of fibrinogen concentration on the clotting time of ancrod, the major thrombin-like enzyme from C. purpureomaculatus, were also determined (Table 2).

3.5. The clotting activity of thrombin-like enzymes on fibrinogen from various animals

The clotting activity of purpurase and ancrod on the fibrinogens of human, cat, dog, goat and rabbit showed some distinct species differences (Table 3). The clotting time of fibrinogen by purpurase in the decreasing order: cat > human > dog > goat >> rabbit (no clot), while that of ancrod was: goat > cat > dog > human > rabbit.

3.6. The release of fibrinopeptides by purpurase

The fibrinogenolytic activity of purpurase was examined using a 1% bovine fibrinogen solution. The reversed-phase HPLC profile of the separation of the incubation mixtures of fibrinogen with purpurase, ancrod and thrombin was shown in Fig. 3. As expected, thrombin released fibrinopeptide A and B after 10 min incubation, and ancrod only released fibrinopeptide A. Under the same conditions, purpurase only released fibrinopeptide A after 2 h of incubation. However, crude C. purpureomaculatus venom released both fibrinopeptides A and B.

3.7. ELISA cross-reactions

Table 4 shows that in the indirect ELISA, there were no cross-reactions between IgG anti-purpurase and ancrod, but rather strong cross-reactions between IgG anti-purpurase with venoms from Trimeresurus complex, (68–150%). There were also moderate cross-reactions with venoms from the related Protobothrops, Ophophis, Agkistrodon genera (16–71%) and Bothrops genus (41–44%), but low degree of cross-reactivity for C. rhodostoma and the vipers venoms (<10%). There were no cross-reactions between anti-purpurase and the elapid venoms tested. In the double-sandwich ELISA; however, IgG anti-purpurase only cross-reacted with venoms from the Trimeresurus complex (Table 4). It exhibited either very low or zero cross-reactions with ancrod and venoms from other genera.

4. Discussion

4.1. Characterization of purpurase

Purpurase is a single-chain protein with a molecular weight of 35,000, as determined by SDS-PAGE and 38,760 by gel filtration
chromatography. The molecular weight determined is comparable to that of many known thrombin-like enzymes.

Sequence comparison of the N-terminal region of purpurase with several other venom thrombin-like enzymes (Table 5) shows high degree of homology; in particular, purpurase shares 100% sequence homology with catroxobin (Pirkle et al., 1989). On the other hand, the N-terminal sequence of purpurase exhibited a low degree of homology (<30%) to thrombin, kallikrein and trypsin.

Fig. 3. Reversed-phase HPLC profiles of fibrinopeptides released on incubation of fibrinogen with thrombin and thrombin-like enzymes. Enzyme or venom (10 μg) was added to 600 μL bovine fibrinogen (1% in saline) and incubated at 37 °C. Aliquots of the reaction mixture were withdrawn at 10 min (thrombin, A; ancrod, B) or 120 min (purpurase, C; C. purpureomaculatus venom, D) and boiled for 5 min to denature the enzyme. The clots that were formed were removed by centrifugation at 8000 × g for 10 min. The supernatant containing fibrinopeptides were analyzed by reversed-phase HPLC. The sample (100 μL) was loaded on a C18 column (0.4 × 22 cm), and eluted by a linear gradient (0–100%) between solvent A (25 mM ammonium acetate, pH 6.0) and solvent B (50% acetonitrile in 50 mM ammonium acetate, pH 6.0) at a flow rate of 1 mL/min. Absorbance was monitored at 214 nm.

4.2. Arginine esterase and amidase activities of purpurase

Like other venom thrombin-like enzymes, purpurase exhibited both arginine ester hydrolysis and amidase activities. Tripeptide chromogenic anilide substrates have been used to study the specificity of thrombin-like enzymes (Lotternberg et al., 1981; Cho et al., 1984; Magalhaes et al., 2006). Many of these tripeptide substrates were designed based on the amino acid sequences around the cleavage sites.
in fibrinogen or several zymogens of the coagulation systems. In the present study, four tripeptide nitroanilides were used to investigate the specificity of purpurase: N-p-tosyl-Gly-Pro-Lys-nitroanilide, a substrate used for assay of plasmin, N-benzoyl-Phe-Val-Arg-p-nitroanilide and N-benzoyl-Pro-Arg-p-nitroanilide, both excellent substrates for thrombin, and N-benzoyl-Val-Gly-Arg-p-nitroanilide, a good substrate for Factor Xa. The kinetic results show that purpurase is not fastidious towards its substrate. N-p-tosyl-Gly-Pro-Lys-p-nitroanilide is the best substrate, but the \( K_m \) is only slightly higher than the other three substrates examined. It is interesting to note that the \( K_m \) of N-benzoyl-Phe-Val-Arg-p-nitroanilide is comparable to that of N-benzoyl-Pro-Arg-p-nitroanilide, which was not hydrolyzed by gabonase (Pirkle et al., 1986). In contrast, Magalhaes et al. (2006) reported that for the thrombin-like enzyme from Lachesis muta muta, the \( K_m \) for N-benzoyl-Phe-Val-Arg-p-nitroanilide was 66 times higher than that for N-benzoyl-Pro-Arg-p-nitroanilide. The differences are due to the differences in substrate binding subsites of the enzymes.

### 4.3. Fibrinogen clotting activity of purpurase

For the clotting activity of purpurase, the optimal concentration range of fibrinogen was rather narrow, the coagulant action was inhibited when fibrinogen concentration was below 1 mg/mL, or exceeded 3 mg/mL. Similar observations have been reported by Pirkle et al. (1986). In contrast, ancrod has a broader optimal concentration range between 1 to 8 mg/mL.

The clotting activity of purpurase showed some distinct species variation and its species specificity is somewhat different from that of ancrod. However, both purpurase and ancrod, as well as many other venom thrombin-like enzymes, are active versus human fibrinogen, while rabbit fibrinogen was the most resistant. These findings reflect the differences in the structure of fibrinogen from different species as well as the specificity of the various venom thrombin-like enzymes.

### 4.4. The release of fibropeptides by purpurase

Unlike thrombin, many venom thrombin-like enzymes (for example, ancrod, batroxobin and flavoxobin) only release fibropeptide A from fibrinogen (Holleman and Coen, 1970; Stocker and Barlow, 1976; Shieh et al., 1988). However, certain venom proteinases such as gabonase and cerastobin split first the \( \alpha \)a and then the \( \beta \)b chain of fibrinogen. In this study, reversed-phase HPLC analysis of the products showed that purpurase released fibropeptide A only. It is interesting that the crude \( C. \) purpureomaculatus venom released both fibropeptides A and B when incubated with fibrinogen. This may be due to the presence of other types of thrombin-like enzymes or \( \beta \)-fibrinogenase in the venom.

### 4.5. ELISA cross-reactions of purpurase

The extensive indirect ELISA cross reactions between anti-purpurase and many crotalid venoms indicated that many crotalid thrombin-like enzymes exhibit similar antigenic determinants, and that antivenoms raised against crotalid venoms may exhibit parasppecific protection against thrombin-like activity of other crotalid venoms. An interesting exception is \( C. \) rhodostoma, another common pit viper in the same geographical region: there is little cross-reaction between anti-purpurase and \( C. \) rhodostoma venom, or its major thrombin-like enzyme, ancrod. The results suggest that there is a significant difference in the protein structure between ancrod and the thrombin-like enzyme from other common crotalid venoms. The lack of indirect ELISA cross-reactions between anti-purpurase with viperid and elapid venoms is not surprising, as both viperid and elapid venoms generally do not exhibit thrombin-like enzyme activity.

In the double-sandwich ELISA, anti-purpurase generally yielded much lower levels of cross-reactions with other venoms. The higher specificity of double-sandwich ELISA is an established phenomenon (Tan et al., 1993). It is interesting to note that of the wide range of venoms tested, anti-purpurase only cross-reacted with certain venoms from the \( Trimeresurus \) complex but not with venoms from other crotalids. This cross-reactivity pattern supports the recent proposed alterations in taxonomy of the \( Trimeresurus \) complex. Previously, all the 40+ species of Asian lance-head pit vipers were grouped into the genus \( Trimeresurus \). However, recently, the taxonomy of this genus has been modified (Kraus et al., 1996), and many authors agreed that the species formerly assigned to the genus \( Trimeresurus \) are to be arranged in four genera: \( Trimeresurus sensu stricta \) (s.s.), \( Ovophis \), Proteothrobothrops and Trapidolaothes (Malhortra and Thorpe, 2002). Our double-sandwich ELISA studies showed that anti-purpurase cross-reacted with all venoms of species from the \( Trimeresurus sensu stricta \) (the \( C. \) retypeorthops, \( P. \) parvipes, \( V. \) dariovipera, \( T. \) persimilis) but not with venoms of species from the Proteothrobothrops (\( P. \) flavoviridis, \( M. \) macrospunatus, \( P. \) elegans, \( P. \) tokaravensis), Trapidolaothes wagleri and \( Ovophis \) okinavenisis. The results confirm that the species in \( Trimeresurus sensu stricta \) are indeed distinct species of purpurase:

### Table 4

ELISA cross-reactions of anti-purpurase.

<table>
<thead>
<tr>
<th>Venoms</th>
<th>Indirect ELISA (% cross reaction)</th>
<th>Double-sandwich ELISA (% cross reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimeresurus complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptelytrops purpureomaculatus</td>
<td>148.1 ± 2.6</td>
<td>114.6 ± 7.2</td>
</tr>
<tr>
<td>Cryptelytrops sibulobatis</td>
<td>1449.3 ± 3.6</td>
<td>100.1 ± 2.0</td>
</tr>
<tr>
<td>Cryptelytrops macropa</td>
<td>1348.3 ± 3.8</td>
<td>42.0 ± 3.7</td>
</tr>
<tr>
<td>Cryptelytrops erythrurus</td>
<td>984.6 ± 3.4</td>
<td>40.1 ± 1.8</td>
</tr>
<tr>
<td>Paras sumatrana</td>
<td>1116.6 ± 3.5</td>
<td>182.2 ± 2.7</td>
</tr>
<tr>
<td>Pepsis papeorum</td>
<td>670.4 ± 4.7</td>
<td>695.4 ± 4.4</td>
</tr>
<tr>
<td>Viridivipera stejnegeri</td>
<td>884.4 ± 5.8</td>
<td>518.3 ± 5.3</td>
</tr>
<tr>
<td>Trimeresurus gramineus</td>
<td>1334.4 ± 3.0</td>
<td>45.6 ± 5.7</td>
</tr>
<tr>
<td>Protothochoerus flavoevidris</td>
<td>713.3 ± 2.8</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Protothochoerus macrouquamaus</td>
<td>363.3 ± 4.7</td>
<td>0</td>
</tr>
<tr>
<td>Protothochoerus elegans</td>
<td>416.4 ± 2.2</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>Protothochoerus tokaravensis</td>
<td>372.2 ± 4.7</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>Ovophis okinavenisis</td>
<td>33.1 ± 3.7</td>
<td>0</td>
</tr>
</tbody>
</table>

### Others venoms

<table>
<thead>
<tr>
<th>Venoms</th>
<th>Indirect ELISA (% cross reaction)</th>
<th>Double-sandwich ELISA (% cross reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calloselasma rhodostoma</td>
<td>3.8 ± 2.2</td>
<td>0</td>
</tr>
<tr>
<td>Tropidolaemus wagleri</td>
<td>8.7 ± 2.8</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Glycidus blomhoffi blomhoffi</td>
<td>73.1 ± 2.6</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>Agkistrodon bilineatus bilineatus</td>
<td>68.7 ± 2.7</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Agkistrodon contortrix contortrix</td>
<td>610.4 ± 4.3</td>
<td>0</td>
</tr>
<tr>
<td>Bothrops asper</td>
<td>444.3 ± 3.7</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>Bothrops atrox</td>
<td>413.4 ± 4.3</td>
<td>0</td>
</tr>
<tr>
<td>Crotalus adamanteus</td>
<td>63.7 ± 2.8</td>
<td>0</td>
</tr>
<tr>
<td>Echis carinatus</td>
<td>605.4 ± 4.2</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>Viperia ammodytes ammodytes</td>
<td>9.4 ± 3.0</td>
<td>0</td>
</tr>
<tr>
<td>Debra russelli slaimens</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Noja nigricollum</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Noja spastrix</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bungurus fasciatus</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Thrombin-like enzymes

<table>
<thead>
<tr>
<th>Venoms</th>
<th>Indirect ELISA (% cross reaction)</th>
<th>Double-sandwich ELISA (% cross reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancrod</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Purpurase</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

The absorbances of the reaction between purpurase and IgG anti-purpurase at 402 nm were 1.04 ± 0.03 and 1.03 ± 0.03, respectively, for indirect ELISA and double-sandwich ELISA. Values were the mean of 6 determinations.

### Table 5

N-terminal amino acid sequences of purpurase and other serine proteinases.

<table>
<thead>
<tr>
<th>Serine Proteases</th>
<th>N-terminal Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purpurase</td>
<td>V V G D E C N I H E R S L V A I F</td>
<td>This work</td>
</tr>
<tr>
<td>Catroxoobin</td>
<td>V V G D E C N I H E R S L V A I F</td>
<td>Pirkle et al., 1989</td>
</tr>
<tr>
<td>Flavoxobin</td>
<td>V V G D E C N I H P L V A L C</td>
<td>Shieh et al., 1988</td>
</tr>
<tr>
<td>Crotalase</td>
<td>V V G D E C N I H E R S L V A I F</td>
<td>Pirkle et al., 1981</td>
</tr>
<tr>
<td>Gabonase</td>
<td>V V G D E C N I H E R S L V A I F</td>
<td>Pirkle et al., 1986</td>
</tr>
<tr>
<td>Thrombin</td>
<td>I V V G D E C N I H E R S L V A I F</td>
<td>Magnusson et al., 1975</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>V V G Y C E H E N S Q P W V A Y V</td>
<td>Swift et al., 1982</td>
</tr>
<tr>
<td>Trypsin</td>
<td>V V G Y C E H E N S Q P W V A Y V</td>
<td>Titani et al., 1975</td>
</tr>
</tbody>
</table>
very different from Protobothrops, Ovophis and Tropidolaemus. It is interesting that results from immunological cross-reaction studies support the taxonomic conclusion based on studies on mitochondrion genes.

Acknowledgments

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References


