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A Preliminary Study for the Detection of Gelatinolytic Proteases from the Scorpion *Androctonus crassicauda* (Turkish Black Scorpion) Venom

[*Androctonus crassicauda* (Türk siyah akrebi) akrep venomunda jelatinolitik proteazların belirlenmesi için bir ön çalışma]

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ABSTRACT

Objectives: Despite the long history on proteolytic activity studies, no study has been reported about the presence of proteases from the Turkish scorpion *A. crassicauda* venom which is among the most toxic species in the world. The present work is a preliminary study indicating the presence of gelatinolytic proteases in the *A. crassicauda* crude venom which almost nothing is known about its components.

Methods: Protein contents of the venom were analyzed by electrophoresis. Further, the venom fractions were separated by liquid chromatography. Proteolytic activities of crude venom and each fraction were determined by zymogram assay.

Results: Two proteins with gelatinolytic activity were detected between 36-45 kDa and 97-116 kDa in the soluble venom at a range of pH 4.0 to 8.0. Proteolytic bands from the fractions obtained chromatographically were detected as three bands between 14.2 and 20 kDa; one band between 20 and 24 kDa; three bands between 24 and 29 kDa; two bands between 97 and 116 kDa at pH 7.

Conclusion: Here, first electrophoretic separation of the venom from *A. crassicauda* where the proteolytic activity against gelatin is reported. These enzymes may prove to be a useful tool for the study of toxin processing and understanding the mechanism of venom activation.

Key Words: *Androctonus crassicauda*, Turkish black scorpion, venom, protease, chromatography, gelatin.

ÖZET

Amaç: Proteolitik aktivite çalışmalarının uzun geçmişine karşılık, dünyanın en toksik türleri arasında olan Türk akrebi *Androctonus crassicauda* venomunun içerdiği proteazların varlığı üzerine bir çalışma bildirilmemiştir. Bu çalışma, venom bileşenleri hakkında literatürlerde yok denecek kadar az bilgi olan *A. crassicauda* ham venomunda jelatinolitik proteazların bulunduğunu gösteren bir başlangıç çalışmasıdır.

Metot: Venomun protein bileşenleri elektroforez ile analiz edilmiştir. Ayrıca, venom fraksiyonları sıvı kromatografide ayrılmıştır. Ham venomun ve her bir fraksiyonun proteolitik aktiviteleri zimogram çalışması ile belirlenmiştir.

Bulgular: pH 4.0 ile pH 8.0 arasında çözülebilir *A. crassicauda* venomunda, 36-45 kDa ve 97-116 kDa arasında olan jelatinolitik aktiviteli iki protein belirlenmiştir. pH 7'de, kromatografik olarak elde edilen fraksiyonlardan, proteolitik bandlar 14.2 ve 20 kDa arasında üç band; 20 ve 24 kDa arasında bir band; 24 ve 29 kDa arasında üç band ve 97 ve 116 kDa arasında iki band olarak tesbit edilmiştir.

Sonuç: Bu çalışma ile jelatine karşı proteolitik aktivitenin olduğu *A. crassicauda* venomundan ilk elektoforetik ayırım bildirilmektedir. Bu enzimlerin toksin ile ilgili süreçlerin çalışılması ve venom aktivasyon mekanizmasının anlaşılması için yararlı araçlar olabileceğini göstermektedir.

Anahtar Kelimeler: *Androctonus crassicada*, Türk siyah akrebi, venom, proteaz, kromatografi, jelatin

Introduction

At least sixteen different species of scorpions compose the scorpion fauna of Turkey and they belong to four families: Buthidae, Euscorpiide, Iuridae and Scorpionidae [1]. *Androctonus crassicauda* belongs to the Buthidae family and it is well known that the sting of scorpions of such family is dangerous to humans, because their venom contains neurotoxins that recognize mammalian receptors [2].

A. crassicauda is a wide spread species in the Middle East. Its distribution includes Turkey (Southern Anatolia), Iran, Iraq (Mosul), Syria (Palmyra, Homs and Damascus), Jordan and Arabia [3]. Stings caused by *A. crassicauda* scorpions are known to cause human envenomation that requires medical attention, some of which might be fatal [4]. To date only two toxic peptides, named Acra1 and Acra2, have been isolated and chemically characterized from this venom and few is known about their venom activity [5].

Previous studies have shown that scorpion venoms are a rich source of toxic polypeptides with a variety of pharmacological functions, especially those that affect membrane permeability for Na^+, K^+, Ca^{2+} and Cl^- of excitable and non-excitable cells. In addition to these basic polypeptides, scorpion venom have been found to contain some enzymes, such as phospholipase A2, hyaluronidase, acetylcholinesterase, alkaline phosphatase and proteolytic enzymes with gelatinolytic activity [6, 7]. It was suggested that proteolytic enzymes cause necrosis, hemolysis and gangrene following scorpion stings [8]. However, these symptoms had not been reported in humans after the stung of *A. crassicauda* [9]. Additionally in a comparative study of the enzymes in different scorpion species, it has been proven that proteolytic enzymes are present in the venoms of the buthid scorpions *Leiurus quinquestriatus* and *Heterometrus spinnifer.* Also proteolytic enzymes are present in low levels in the venoms of the scorpions *Buthotus judaicus*, *Parabuthus transvaalicus*, *Scorpio maurus palmatus*, *Androctonus australis* and *Pandinus imperator* [10].

Almost nothing is known about components of *Androctonus crassicauda* crude venom up to date. Here, for the first time, we were given evidence of proteolytic enzymes with gelatinolytic activity in the crude venom of *A. crassicauda* as a preliminary study. The venom separation was performed by means of high performance liquid chromatography (HPLC). Gelatinolytic activity of the crude venom and the purified protein fractions are shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with co-polymerized gelatin assay under different pH conditions.

Materials and Methods

Scorpion collection and venom extraction

Specimens of *A. crassicauda* (Oliver, 1087) were collected in rural areas of the state of Sanliurfa, Turkey. They were kept alive in plastic boxes in the laboratory and fed with crickets and water at *libitum*. Total venom was collected by electrical stimulation of the animals. The crude venom was dissolved in distilled water and centrifuged at 14,000 x *g* for 15 min. The supernatant containing the soluble fraction of the venom was aliquoted, freeze-dried and stored at -20 ^{o}C until use as described previously [5]. All chemicals were analytical grade reagents, obtained from Sigma-Aldrich Co. Ltd.

Gel electrophoresis

To determine protein profile of *A. crassicauda* crude venom, SDS gel electophoresis was performed. Samples were submitted to a 5% stacking and 12% resolving polyacrylamide gel electrophoresis under non-reducing and reducing conditions. Gel electrophoresis was performed at room temperature, 120 V, approximately for 2 h. After electrophoresis, the gel slabs were stained with Coomassie brilliant blue R-250 in 25% isopropanol and 10% acetic acid and subsequently were destained in a 10% methanol and 5% acetic acid solution [11]. For the analyses of small proteins, crude venom was run on Tris Tricine SDS-PAGE using a 10% resolving and 4% staking gel solutions [12].

Separation procedures

The lyophilized venom (2 mg) was separated by HPLC system (SHIMADZU), using a prominence model, equipped with a photodiode array detector and a C18 reverse column (Vydac; Hisperia, CA, USA). The column was equilibrated with 0.12% TFA (solvent A) and eluted in a 0 to 60% gradient of solvent B (acetonitrile/0.1% TFA), at a flow rate of 1.0 ml/min. for 60 min. [13]. Elution was monitored by UV absorbance at 230 nm simultaneously. Fractions were collected in 5 ml plastic tubes, dried out under vacuum and protein content of fractions was calculated by the absorbance at 280 nm [14]. The fractions were subject to gelatinolytic assay.

SDS-PAGE-zymograms under different pH conditions

To study the proteolytic activities in the venom and its fractions, zymogram assays were performed by 12% acrylamide containing 1 mg/ml of bovine skin gelatin as a substrate in 0.1% SDS, 0.4 M Tris-HCl buffer pH 8.8 [8, 11]. A stacking gel, consisting of 5% polyacrylamide 0.1% SDS in 0.06 M Tris-HCl buffer at pH 6.8 was also used. Samples were applied onto the gel under non-reducing conditions and electrophoresis was performed for 3 h at 4 ^{0}C and at a constant current voltage of 100 V. After electrophoresis, the gel slabs were washed in 2.5% Triton X-100 for 30 min to remove SDS. The gel was then washed in distilled water and incubated overnight at 37 ^{0}C in desired buffers to determine proteolytic activity. For pH 4.0, 5.0 and 6.0, the gel was developed in 50 mM sodium citrate buffer. For pH 7.0, 8.0 and 9.0 the

gel was developed in 50 mM Tris-HCl buffer. For pH 10, the gel was developed in 10 mM carbonate-bicarbonate buffer and after incubation; the gels were stained and destained as described above. Clear zones of substrate lysis against a blue background stain indicated the presence of gelatin-degrading enzymes. Inhibition of the proteolytic activity of a venom fraction was determined by a zymogram assay using 50 mM Tris-HCl buffer, pH 7.4 in the presence of 0.002 mM aprotinin, 10 mM EDTA (ethylene diamine tetra acetic acid) containing 5 mM $CaCl_2$, and 10 mM PMSF (phenyl methyl sulphonyl fluoride) [15,16]. As a control, the venom fraction was developed in the absence of any protease inhibitor in a parallel well. Band Leader software version 3.0 was used to estimate the relative intensities for lysed gelatin zones from zymographs [17].

Results

The soluble venom of *A. crassicauda* was analyzed by SDS-PAGE under different conditions (Figure 1). Multiple protein bands ranging from 6 to 200 kDa were observed in the venom analyzed under non-reducing (Figure 1A) and reducing (Figure 1B) conditions. Reducing and non-reducing samples were presented quite different band profile. Two proteins between 36-55 kDa and five proteins between 6.5-29 kDa were disappeared on the reducing gel. On the other hand one protein band close to 45 kDa, two protein bands between 29-36 kDa and a 6.5 kDa protein band were appeared as new bands on the reducing gel.

In order to analyze the densely stained protein band at the lower molecular weight, samples were further analyzed by Tris Tricine SDS-PAGE system in both non-reducing and reducing conditions (Figure 1C). The strong band between 6.5 and 14.5 kDa on the previous non-reducing SDS-PAGE gel was observed as two bands in Tris Tricine SDS-PAGE non-reducing gel. These two bands were reduced to a smaller band in the reducing Tris Tricine SDS-PAGE gel system, similar to the result obtained from SDS-PAGE reducing gel.

Results of the gelatin zymogram experiments with crude venom in different pH conditions are presented in Fig. 2A. The major proteolytic activity was observed with a colorless band in the pH 6-8 range and it corresponds to a molecular weight between 36 and 45 kDa. Also weak proteolytic activities were observed at pH 7 and pH 8 in the protein bands between 97 and 116 kDa. Additionally, zymographs were quantified with the aid of the Band Leader software and the major venom proteases possess a pH range from acidic to neutral conditions (Figure 2B and 2C).

The proteins of the venom from *A. crassicauda* were separated by RP-HPLC and chromatographic profile is shown in Fig. 3. The fractions obtained were named as from F1 to F14 and their proteolytic activities were determined in SDS-PAGE gelatin assay at pH 7 where

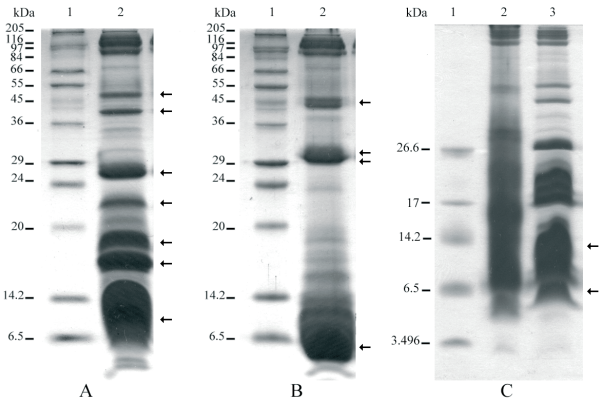

Figure 1. Analyses of crude venom from *A. crassicauda* by 12% SDS-PAGE under non-reducing (A) and reducing conditions (B). Lane 1: molecular weight markers, and lane 2: *A. crassicauda* crude venom. (C) Analyses of crude venom by 10% Tris-Tricine SDS-PAGE under reducing (line 2) and non-reducing (line 3) conditions.

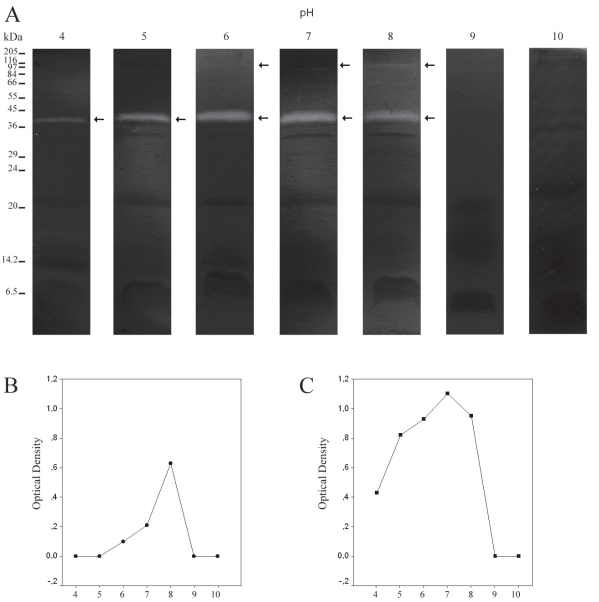

Figure 2. Protease activities of *A. crassicauda* crude venom (100 µg/lane) were analyzed by 12% SDS-PAGE containing co-polymerized bovine skin gelatin under different pH conditions. Zymograms were developed overnight at 37^0C in the presence of different buffers and pH values (indicated at the top of the lanes) as described in materials and methods, proteolytic activity was observed as colorless bands (A). Molecular weight markers are shown on the left. Protein bands were densitometrically quantified and graphically plotted for 97-116 kDa (B) and 36-45 kDa (C). Proteolytic activities were reported as optical densities (ordinate) in function of pH (abscissa).

the strong clear band of the crude venom proteases is observed.

The proteolytic activities of the fractions were observed as three bands between 14.2 and 20 kDa for F1, F3 and F4; one band between 20 and 24 kDa for F6; three bands between 24 and 29 kDa for F10, F11 and F13; two bands between 97 and 116 kDa for F3 and F4 at pH 7. Proteolytic activity was not detected for other fractions; F2, F5, F7, F8, F9, F12 and F14 (Figure 4 A and B). Among them

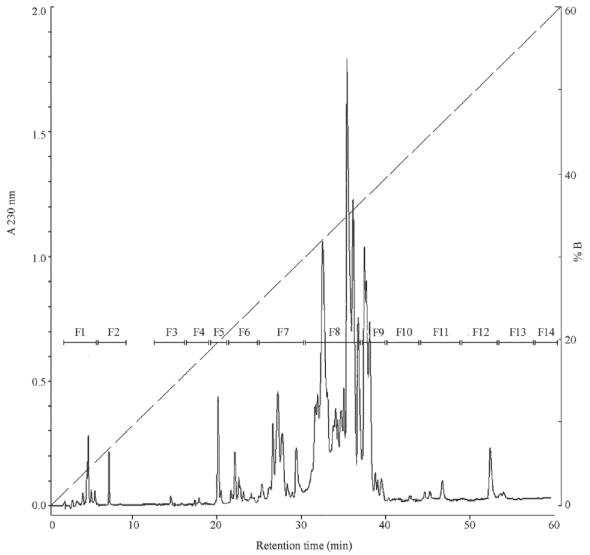

Figure 3. Chromatographic separation of *A. crassicauda* venom. 2 mg of the soluble venom from *A. crassicauda* was fractioned using a reverse-phase C_{18} analytical column equilibrated in 0.12% TFA, and eluted with a linear gradient of acetonitrile from 0 to 60% in 0.1% TFA, run for 60 min at flow rate of 1 ml/min. The fractions collected were named F1 to F14 as indicated.

F6 was selected as a major proteolytic band, and then examined further for its sensitivity to different protease inhibitors. After incubation of the fraction with EDTA (metalloproteinase inhibitors), aprotinin and PMSF (serine protease inhibitors), here was no detectable protease activity at all, as depicted in Figure 4 C.

Discussion

A. crassicauda is well known as one of the most toxic scorpions in the world [18]. This specie is responsible for human envenomation especially in Southeastern Anatolia of Turkey [4]. In 2003, 598 cases were documented in Sanliurfa province, between May and September, and 299 people were stung by *A. crassicauda* [19]. Also, around 50.000 scorpion stings are reported every year and 30% of cases are caused by *A. crassicauda* with a rate of mortality of 1.5% in Iran [20]. Up to date, very little is known about the function of peptide toxins of this specie of scorpion. Here, we investigated the proteolytic properties of *A. crassicauda* crude venom and its components. Electrophoretic analysis confirmed that the crude venom of *A. crassicauda* contains numerous proteins with gelatinolytic properties.

Several proteases from various venom sources such as scorpion, snake, bee, spider and centipede have been isolated and well studied [21-24]. Activity of these proteases on various proteins such as casein, gelatin, hemoglobin, immunoglobulin, albumin, fibrinogen, fibronectin and laminin has been well documented [15, 16]. Here, we found that *A. crassicauda* crude venom proteases are active on gelatin under physiological

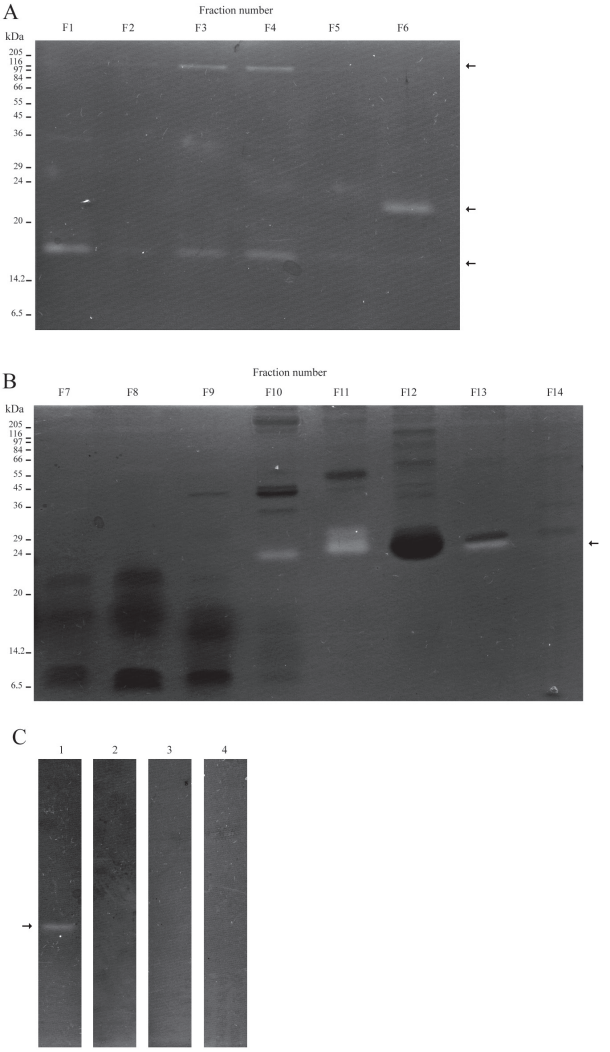

Figure 4. The proteolytic activity of the venom fractions (50 µg/lane) from *A .crassicauda* was analysed by 12% of SDS-PAGE containing 0.1% gelatin. (A) Fractions from F1 to F6 and (B) F7 to F14. Molecular weight markers are shown on the left. (C) Inhibition of F6 at pH 7.4 by aprotinin (line 2), EDTA (line 3) and PMSF (line 4). Line 1 indicates the activity of F6 as a control.

conditions (pH ~7.0). Therefore it may be active on critical molecules or tissue structures in envenomated people. Although crude venom showed strong proteolytic activity between 36-45 kDa, none of the fractions showed activity in this molecular weight range. However, a band between 36-45 kDa was strongly observed as a dark non-active band in only F10 fraction on the zymogram (Figure 4B). We surmised that these may be the same protein band and the differences between their activities are very likely due to the instability of the enzyme by the TFA treatment during HPLC separation. Zymograms performed with the fractions showed several proteolytic bands, whereas crude venom exhibited only two bands, because of the high concentration of the proteins in the fraction samples.

Analysis of F6 as a major proteolytic protein with different protease inhibitors indicated its specificity. Proteolytic activity of the fraction was inhibited by three inhibitors used in the gelatin assay. Among them EDTA has known as a metalloproteinase inhibitor, aprotinin and PMSF as serine protease inhibitors. Even the inhibitory activity of EDTA was supported by the addition of $CaCl_2$, since EDTA binds excess Ca^{2+} and inhibits enzyme activity [25]. Therefore, the results obtained here indicate that F6 has a serine metalloproteinase activity. Recently, Berger et al. (2008) also showed a serine metalloproteinase activity of a protease from snake venom, using EDTA and PMSF as an inhibitors [26]. The best of our knowledge, here the first time we determined a serine metalloproteinase from scorpion venom.

Present study shows that at least 10 different proteolytic peptides can be found in *A. crassicauda* crude venom. Some reasons for the presence of proteolytic enzymes in animal venoms could be that venom proteases may be responsible for some pathological activities triggered by these venoms and also contribute to the local and systemic symptoms observed in envenomations [16]. Protease activities may point to venom act as spreading factor to the body of the victims and increase tissue permeability to make easy the spread of proteins in the whole venom [22, 27]. Additionally, these enzymes may be involved in the post-translational processing of toxins and then activation of venom components. Previous studies have suggested that proteolytic enzymes were present based on observations of necrosis, hemolysis, gangrene, pancreatitis and severe acute respiratory syndrome following scorpion envenomation [8, 28]. Clinical reports indicate that *A. crassicauda* scorpion stings can cause severe pain, autonomic central nervous system diseases, muscle function disturbances, respiratory diseases and death [19, 20, 28, 29]. Interestingly, no any case reported describing proteolytic enzyme symptoms except severe acute respiratory syndrome in human stung by this species. The case report on *A. crassicauda* envenomation proves that spontaneous breathing of victims was stopped [30]. The reason for insufficient knowledge for other symptoms may be due to the lack of records on *A. crassicauda* sting.

In conclusion, here the first electrophoretic separation of the venom from Turkish scorpion *A. crassicauda* where the proteolytic activity against gelatin is reported. Among the components, at least 10 proteolytic enzymes were determined. Most importantly, first time we indicated the presence of a peptide toxin with a serine metalloproteinase activity in *A. crassicauda* scorpion venom. The biological function of the proteolytic enzymes in *A. crassicauda* venom is currently unknown but these are still under investigation. Proteases are well known spreading factors and *A. crassicauda* proteases may be useful tools for the future research of cell biology, biochemistry and pharmaceutical industry.

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