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Proteolytic Profile Alterations as One of the Scorpion's *Leiurus macroctenus* Envenomation Effects on Kidneys

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Abstract

Scorpion envenomation becomes a serious challenge for the healthcare systems in tropical and subtropical regions. Among the variety of organs being affected by the venom, kidneys can accumulate most of the toxins recently after the sting, therefore, the homeostasis, including the proteolytic homeostasis of the renal system during the envenomation is under question. Using the SDS-PAGE and zymography methods we have investigated the proteolytic profile in the rats' kidneys during the *Leiurus macroctenus* scorpion envenomation. As it turned out, envenomation leads to the increase of the relative activity of enzymes with collagenolytic, gelatinolytic and fibrinogenolytic properties with molecular weights of 30-50 kDa and 50-70 kDa, simultaneously decreasing the relative activity of proteases with higher molecular weight (70-100 kDa). We have also observed the major changes occurring in 24 hours after the envenomation. We may assume that the obtained results are most likely related either to the formation of proteolytically active fragments of proteases with higher mass, or the excessive expression of proteases with lower mass, yet additional tests need to be conducted to prove these hypotheses. Significant changes in assessed parameters in 24h after the envenomation suggest the increased danger in this period of envenomation for the proteolytic homeostasis in kidneys and the integrity of the renal system overall. Therefore, the described effects can be an important reason for the kidney dysfunctions during the *Leiurus macroctenus* envenomation.

Keywords: Envenomation, Enzymes, Kidneys, Proteolytic Activity, Scorpion

1. Introduction

One of the common public health problems in tropical and subtropical regions is scorpion envenomation. Not enough attention is being paid to scorpionism in many countries (Middle East, Brazil, India in particular), mostly due to the mild clinical manifestations and undeveloped healthcare systems. Nevertheless, the estimated number of scorpion stings amounts to up to 1.2 million annually,

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including severe and moderate envenomation cases, that lead to patient health complications or even death (about 3,250 cases per year)[1].

Scorpion venom is mostly comprised of peptides (including the ion-channel blockers), ions, biogenic amines and enzymes. The latter plays a critical role in venom spreading throughout the body. Proteolytic enzymes, mostly metalloproteases and serine proteases, can degrade extracellular matrix proteins and blood vessel walls, "clearing the path" for the venom components, moreover, they can activate venom toxin precursors, potentiating the venom dangerousness[2].

The *Leiurus* genus comprises one of the most dangerous scorpions around the globe, mostly populated in the Middle East and North Africa regions. The clinical manifestations of *Leiurus* scorpion envenomation include mostly local symptoms, yet systemic complications such as vomiting, headache, and cardiac and pulmonary failure may occur[3]. However, in addition to the neglected attitude to the non-severe cases of envenomation by these scorpions, not enough attention is also being paid to the changes in the patient's organism on the biochemical level. *Leiurus macroctenus* is a relatively new scorpion species, which has been distinguished from other *Leiurus* species by unique morphometric and morphological characters quite recently[4,5]. Despite their danger, there is little information about these scorpions. The missing data on the envenomation features of *Leiurus macroctenus*, including the biochemical level, could be critically important for clinicians in areas where *Leiurus* scorpions are populated.

Recently, Gunas *et al.,* have shown that *Leiurus macroctenus* venom induces the proteolytic activity elevation in the rats' kidneys[6] and causes protein fractions redistribution in this organ as well[7]. Other authors also suggest that the *Leiurus* scorpion envenomation can lead to renal system impairment[8,9]. Taking into account the mentioned information, to expand the knowledge on the *L. macroctenus* venom's effects on the renal system, in this work, we have focused our attention on the relative changes in the proteolytic profile in the envenomated rats' kidneys.

2. Materials and Methods

2.1 Scorpion Collection and Maintenance

In this work, we have used ten mature *Leiurus macroctenus* specimens. All scorpions were previously identified by Mark Stockmann and kept in Ibbenbüren private collection (Germany). Scorpions were kept separately in transparent plastic boxes (ten x five x five cm) layered by one cm with sand (Exo Terra, Desert Sand). In the center of each box were placed bowls with distilled water which were refilled once a week. All animals were maintained in constant conditions: 25°C-35°C, 50-60 % humidity, and natural lighting conditions. Appropriate aeration was achieved by the holes in the container. One *Shelfordella lateralis* cockroach was fed to each scorpion once a week. Containers were cleaned of cockroach remnants once a month.

2.2 Venom Collection

The procedure of scorpion venom obtaining and LD-50 determination was carried out according to *Özkan* and Filazi's method[10], modified by Yaqoob *et al*[11]. Scorpion fixation was followed by the electrode pointing to the scorpion's cephalotorax and telson. An electric current with the intensity of 24 V was applied for five seconds to the base of the telson, while the telson's opposite edge was pointed to the sterile phial. Number of electrode-scorpion contacts varied from one to ten, depending on the amounts of obtained venom. The interval between milking acts was ranged at two weeks. According to the acute toxicity studies, the LD-50 value of the venom was determined as 0.08 mg/kg. The collected venom samples were stored at -20°C.

2.3 Rats' Envenomation Model and Tissue Homogenates' Preparation

The environmental conditions, feeding, cleaning and other procedures related to the experimental rats were provided with the compliance of the Committee for Control and Supervision on Experiments on Animals (CPSEA). To provide statistical relevance, in the experiments were used 73 albino non-linear male rats (180g ± 3g). A group of 60 animals were injected with 0.5 ml venom solution (LD50), dissolved in the saline solution (0.9 %) intramuscularly, and the control group, consisting of 13 rats, was injected with 0.5 l saline solution (0.9 %) alone.

Control and experimental animals were subjected in separate cages by five specimens to the constant vivarium conditions (20-24 °C, 30-70 % humidity, 12h light/dark cycle). The experimental group was randomly divided into four subgroups of 15 animals and the euthanasia of corresponding subgroups was conducted in the time frames of 1 hour, 3h, 24h and 72h after venom injection to evaluate the dynamic changes of the assessed parameters. The euthanasia was performed according to the CPSEA Guidelines, using sodium thiopentone as the anaesthetic agent and cervical dislocation as the euthanasia method.

The kidneys' isolation and homogenization were performed at + 1 – 4°C immediately after the euthanasia. Homogenates were prepared using the 0.05 mol/L Tris-HCl (pH 7.4) buffer with 0.14 mol/L NaCl and 0.001 mol/L Ethylenediaminetetraacetic Acid (EDTA) in the 1:9 tissue-buffer mass proportion. The homogenate samples were centrifuged at 600 g for 15 min and obtained supernatant was centrifuged again at 15,000 g for 15 min. The supernatant was aliquoted to the sterile plastic tubes and stored in liquid nitrogen.

2.4 Serine Proteases' Fraction Purification

Taking into account that benzamidine is a selective inhibitor of trypsin-like proteases, the purification of serine proteases was performed via affinity chromatography on the benzamidine-sepharose column according to the method, used by Raksha *et al*[12]. Homogenate samples were previously dissolved in the 0.01 mol/L Tris-HCl buffer (pH=8.0), containing 0.13 mol/L NaCl in the 1:5 v/v homogenate-buffer proportion. The column was equilibrated by a binding buffer containing 0.02 mol/L Tris-HCl (pH=8.0). The elution of the bound material was carried out by elution buffer containing 0.05 mol/L Glycine-HCl buffer (pH=3.0) with 1 mol/L NaCl. The flow rate of sample loading and elution was set at 2 ml/min. Changes in optical density and conductivity during the purification process were registered by the UV sensor at 280 nm and the conductivity sensor respectively. To get rid of NaCl, the collected fraction was applied to the Superdex G25 column.

2.5 SDS-PAGE

The electrophoretic analysis of serine proteases' fraction was achieved using Laemmli's method of Polyacrylamide Gel Electrophoresis (PAGE) in the presence of Sodium Dodecyl Sulfate (SDS)[13]. Stacking gel and resolving gel, which contained 10 % polyacrylamide, were applied to the electric currents of 19 mA and 36 mA correspondingly utilizing the Mini-PROTEAN Tetra Cell (Bio-Rad, USA) and the PowerPac Basic power supply (Bio-Rad, USA). In the experiment, we have used the SigmaMarker™ wide-range molecular weight markers (Sigma-Aldrich, USA). After separation, the gels were stained and dyed for 15 minutes using the mixture of 2.5% Coomassie Brilliant Blue G-250, 10% ethanol, 10% acetic acid and 15% isopropanol. The excess dye was removed from the gels by placing them in hot distilled water, containing 10% of glacial acetic acid.

2.6 Enzyme Electrophoresis

The enzyme electrophoresis (zymography) was performed to assess the proteolytic profile in the rats' kidneys during the envenomation. The method is based on the standard procedures of SDS-PAGE by Laemmli but with certain modifications[14]. Resolving gels, contained the 12 % polyacrylamide and 1 mg/ml of substrate, namely gelatin, collagen and fibrinogen, stacking gels were prepared according to the standard SDS-PAGE protocols. The conditions of electrophoretic separation were the same as described in the previous subsection. The correspondingly prepared samples of trypsin (23 kDa), mini-plasmin (36 kDa) and plasmin (85 kDa) were used as the molecular weight markers. The gels were incubated for 1h in the 2.5% Triton X-100 solution immediately after the electrophoretic separation to get rid of the SDS. Afterwards, the gels were incubated for 12h at +37°C in the 0.05 mol/L Tris-HCl buffer (pH 7.4) with 0.13 mol/L NaCl. After that, the gels were stained, dyed and washed according to the standard procedure, described in the previous subsection.

2.7 Data Interpretation

The collected data was analyzed and processed via the TotalLab 2.01 and OriginPro v9.5 software. To facilitate and standardize the interpretation of the collected results, the separated protein bands were divided according to their molecular weight into six groups, denoting the molecular weight range: ≤ 30 kDa, 30-50 kDa, 50-70 kDa, 70-100 kDa, 100-150 kDa, ≥150 kDa. The sizes and intensity of lysis bands on zymograms/dyed bands on electrophoregrams within each of the molecular weight groups were expressed as the relative proteolytic activity/relative protein content of each group in % of

total proteolytic activity/protein content. The number of bands within each of the molecular weight groups is also provided on the graphs.

3. Results

The proteolytic activity changes in tissues are closely related to the envenomations due to the presence of venom's proteases as well as the activation of endogenous proteases[6]. In this study, we have examined the changes in the activity of the kidneys' proteases able to utilize collagen, gelatin and fibrinogen as substrates using the enzyme electrophoresis method. As can be seen in Figure 1(A), starting from the 1st hour of envenomation collagenolytic activity of proteases with Molecular Weight (MW) of 30-50 kDa tends to rise, reaching more than 26% of total proteolytic activity in 24h of envenomation, which is almost five times higher than in the control group, when the activity of proteases within 70-100 kDa range was twice as lower as in the control group. In this period was also observed the appearance of the additional protein band within the range of 30-50 kDa, which in turn disappeared in 72h after venom injection. In addition to that, in the period of 3-24 h of envenomation, we observed the appearance of the collagenolytic bands within MW of 50-70 kDa, which were absent in the control animals. Quite similar results were obtained for the enzymes able to degrade gelatin (Figure 1(B)): in 24h the activity of proteases within the 70-100 kDa range was nearly 2,5 times lower compared to the control group, while additional bands within the range of 30-50 kDa, as well as the bands within 50-70 kDa range, can be observed in the period of 3-24 h of envenomation. According to Figure 1(C), the relative activity of enzymes that can degrade fibrinogen with higher MW (70-100 kDa) decreases throughout the envenomation, while the activity of enzymes with lower MW (30-50 kDa) proportionally increases. In addition to that, we have observed the additional protein band within the 70-100 kDa fraction appearing in 24h after venom injection, disappearing in the next 48h.

Previously, we have shown that serine proteases play a substantial role in the proteolytic processes in the rats' kidneys after *L. macroctenus* venom injection[15]. The participation of serine proteases (excluding those present in venom itself) in the scorpion envenomation isn't fully studied. Theoretically, this can be a result of specific activation of zymogens by venom components, or be a part of a more complex mechanism. In this study, we have studied these proteases in the context of *L. macroctenus* envenomation more precisely. We have obtained the serine protease fraction from kidneys homogenate total protein and assessed its protein profile using the SDS-PAGE method, as well as the proteolytic profile via the enzyme electrophoresis method. According to Figure 2(A), *L. macroctenus* venom action in the kidneys results in reduced levels of serine proteases within the MW range

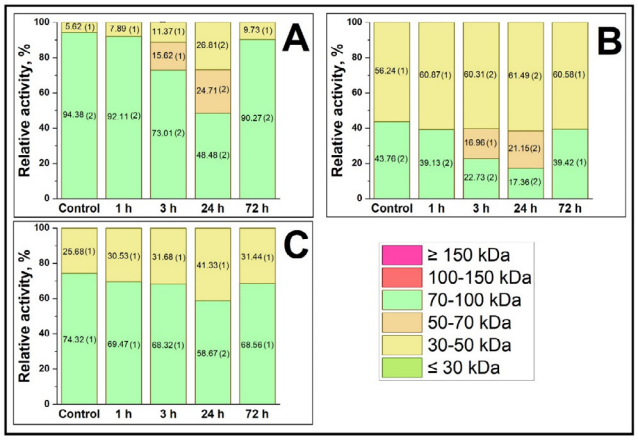

Figure 1. Distribution of relative proteolytic activity among proteases within the given molecular weight ranges, by using as a substrate: **(A)** – collagen; **(B)** – gelatin; **(C)** – fibrinogen. The numerals in brackets display several fractions within each of the protein groups.

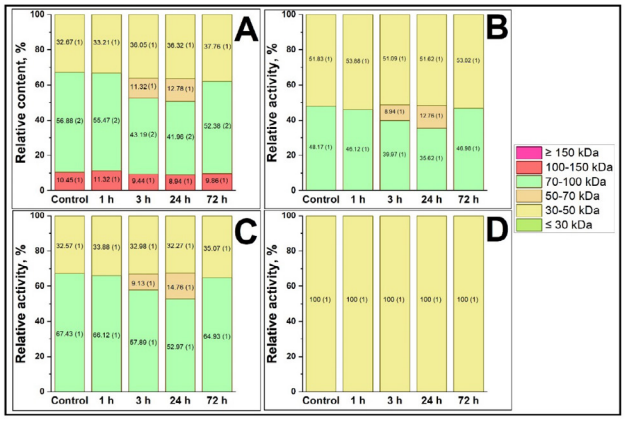

Figure 2. Content and relative proteolytic activity of serine proteases fraction, obtained from kidneys of rats envenomated by *Leiurus macroctenus*. **(A)** – protein profile; **(B), (C)**, and **(D)** – proteolytic profile using as substrates collagen, gelatin and fibrinogen respectively. The numerals in brackets display several fractions within each of the protein groups.

of 70-100 kDa and 100-150 kDa, while the levels of serine proteases with MW of 30-50 kDa have been slightly increasing throughout the envenomation. Moreover, in the period of 3-24 h of envenomation, we have observed the appearance of serine proteases within the MW range of 50-70 kDa. As can be seen in Figures 2(B) and 2(C), the collagenolytic and gelatinolytic activity of serine proteases with MW of 70-100 kDa tend to decrease during the envenomation, when the activity of proteases with MW of 30-50 kDa increase. As in the case of collagenolytic and gelatinolytic profiles of total protein from kidneys homogenate, we have observed the appearance of additional serine proteases within the range of 50-70 kDa from the 3rd to the 24th hour. Nevertheless, we have found no changes in the fibrinogenolytic activity (Figure 2(D)).

4. Discussion

Kidneys are the main organ responsible for the excreting the venom toxins as well as the endotoxins, formed during the envenomation. Notably, most of the venom accumulates in the kidneys, therefore, the renal system is in imminent danger during the envenomation[16]. Approximately up to 10% of scorpion envenomation cases result in acute kidney injury and other pathologies including acute tubular necrosis, acute cortical necrosis and chronic kidney disease[17]. It is known that proteolysis dysregulation may lead to inflammation-related kidney pathologies[18], therefore, we have investigated the proteolytic system changes in the kidneys of rats, envenomated by the *L. macroctenus*. According to the results of the total protein zymography using collagen, gelatin and fibrinogen as substrates, the changes in particular protease fractions' activities indicate a certain imbalance in the proteolytic activity during the envenomation. In addition to that, the additional collagenolytic and gelatinolytic bands within 50-70 kDa in 3-24 h of envenomation also may suggest the proteolytic system dysregulation, as the effect of the envenomation. These results can be explained at least by three potential mechanisms.

Firstly, the pattern of proteolytic activity changes suggests the possible formation of proteases with lower MW from proteases with higher MW via the limited proteolysis mechanism. It is known that many animals' venoms contain enzymes, able to proteolytically activate various zymogens, resulting in the hemostasis disorders[19]. In recent research Gunas *et al.,* have shown that *L. macroctenus* envenomation induces the formation of protein fractions with low MW and simultaneous reduction in the levels of protein with higher MW[7], so it might be possible that the venom can also degrade high MW proteases, resulting in formation of proteolytically active fragments with lower MW. Moreover, it is likely, that the enzymatic activity of these fragments could be higher than in the parent molecule since they might be insensitive to the inhibitors and other regulatory mechanisms.

Secondly, the imbalance in the proteolytic activity could be related to the intense expression of the enzymes within the MW range of 30-50 kDa and 50-70 kDa, which are present in fewer quantities in the control group, thus, the proteolytic activity outburst can be linked to the abnormal number of proteases with certain MW. Sifi *et al.*,[20] point out the ability of the *Androctonus australis hector* scorpion to activate the expression of the Matrix Metalloproteases (MMPs), and in a recent study Gunas *et al.,* have shown that *L. macroctenus* venom can increase the levels of various types of MMPs in the time-dependent manner[6].

The third mechanism may involve the activation of existing cell proteases. It is known that the proteolytic activity of many proteases (such as Ca^{2+}-dependent MMPs or calpains) is closely related to the ion balance inside and outside the cell, for example, Ca^{2+} influx can trigger the activation of cytosol proteases[19]. Scorpions' venoms are comprised of various ion channel blockers[21], therefore, it could be possible that ion disbalance during the envenomation can potentially facilitate the activation of various proteases inside and outside the cell.

The assessment of the serine proteases fraction protein profile has shown an interesting outcome regarding the possible explanation of proteolytic activity increment. According to Figure 2(A), we can observe the typical decrement in the relative levels of serine proteases with higher mass and simultaneous increment in the levels of proteases with lower mass. However, the explanation of these results lies either in the enhanced expression of existing proteases with lower MW or in the formation of fragments of other enzymes (which is more likely), excluding the ion-activation mechanism, explained above. Since the gelatinolytic and the collagenolytic profiles of serine proteases fraction are nearly identical to their protein profile, we can conclude that the enhanced expression and limited proteolysis mechanisms as the main possible explanations of serine protease

proteolytic profile changes. Nonetheless, it still isn't clear if these speculations are fair for the other protease types, the proteolytic activity of which also rises during the envenomation. More precise information on the proteolytic profile imbalance etiology might be available after the additional experiments using other methods, such as chromatography. The definition of mechanisms described in this study *L. macroctenus* venom effects will underpin the problematic field of our future studies.

The significant changes in the rats' kidneys' proteolytic profiles indicate the effect of the *L. macroctenus* venom on the protein homeostasis in this organ. The imbalance in the proteolytic activity profile may lead to excessive proteolysis with unpredicted consequences, for example, intracellular protease network disruption or extracellular matrix degradation. The abnormal activity of proteases may participate in the development of typical renal system pathologies during the envenomation. It is also worth mentioning that the major changes in proteolytic profile, including the appearance of undetectable in control group protease fractions, occur in 24h after venom injection. These results strongly correlate with previous findings regarding the elevation of total proteolytic activity in kidneys in this period[6]. Reis *et al.* also underline that the elevation of toxins concentration in the kidneys 24h after the sting is typical for scorpion venoms[22]. Taking into account these facts, we may conclude that during *L. macroctenus* envenomation, renal system homeostasis and integrity are most likely to be disrupted one day after the sting. Therefore, it's critically important to take into account the proteolytic profile assessment during the scorpionism diagnostics design.

5. Conclusions

To conclude, we have examined the changes in the proteolytic profiles of rats' kidneys during the *Leiurus macroctenus* scorpion envenomation. We have observed the significant decrement of collagenolytic, gelatinolytic and fibrinogenolytic activities of fractions with higher molecular weight with simultaneous increment in the relative activity of proteases with lower molecular weight, which can be the results of the increased expression of corresponding proteases, limited proteolysis of proteases with higher mass, or the specifically increased activity of proteases with lower molecular weight. According to the results of protein profiling and proteolytic profiling of purified serine proteases' fraction, the increased activity of lower-molecular weight enzymes of this type is most likely to be linked to the formation of proteolytically active fragments of the enzymes with higher mass, which can be one of the venom enzymes' effects. It is noteworthy that the most significant changes we have observed in 24h after venom injection, which together with the results of recent studies suggest that this period is particularly dangerous for the renal system overall. The clarification on the mechanisms of kidneys' proteolytic profile changes during the envenomation will be implicated in future studies of *L. macroctenus* envenomation.

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