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Iran J Parasitol

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Original Article

Gelatin Zymography of Major Proteases in Exosomes of *Leishmania major* Promastigotes

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Received 18 Jan 2023
Accepted 11 Mar 2023

Keywords:
Gelatin Zymography;
Exosomes;
Leishmania spp.;
Iran

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Abstract

Background: Enzymatic digestion of extra cellular matrix proteins by proteinases of *Leishmania* promastigotes is a complex process. Hence, studies on functional proteomics of these enzymes can help select these enzymes as possible vaccine candidates or selecting candidates for chemotherapy and immunotherapy. Several proteolytic enzymes are involved in virulence of *Leishmania* spp. These enzymes are mostly serine, cysteine and metalloproteases. We aimed to detect proteases in *Leishmania* promastigote exosomes.

Methods: Serine, cysteine and metalloproteases were investigated in exosomes and lysate of *L. major* promastigote using gelatin zymography. The study was carried out in the Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, in 2021.

Results: Zymography findings of metalloproteinases showed transparent bands, including a 63-kDa glycoprotein (GP63). This glycoprotein is a major surface metalloproteinase. In addition, transparent bands belonged to serin proteases and cathepsin were demonstrated in gels associated to *Leishmania* promastigote lysate and exosomes.

Conclusion: Several metalloproteases, serin proteases and cathepsins were shown in promastigote lysate and exosomes of *L. major*, which could purified and used as fractions for immunodiagnostic.

Introduction

Leishmaniasis is a vector-borne infection in humans and animals such as dogs and rodents. *Leishmania* spp. cause visceral, cutaneous and mucocutaneous diseases. *Leishmania* spp. infect approximately two million humans every year (1). Increases in leishmaniasis cases are due to various factors, including military activities, global tourism and immigration from endemic countries. In the life cycle of protozoa, metacyclic promastigotes are transmitted to mammalian hosts by blood-sucking *Phlebotomus* mosquitoes (2). *Leishmania* pathogenesis is attributed to virulence factors, which enable the parasite to invade its hosts and cause infection. Naturally, *Leishmania* proteinases play critical roles in parasite migration through host tissues. Furthermore, these proteinases degrade proteins associated with immune system to evade immune responses. Promastigotes of *Leishmania* spp. include a major surface protease or GP63 as a virulence factor that enhances parasite survival through modulating macrophage-killing mechanisms (3).

Exosomes are small vesicles secreted by several types of cells, including *Leishmania* cells. They can help transfer various molecules between the cells. Exosomes include cell derived bioactive molecules such as proteins and RNAs (e.g., microRNAs) and play important roles in regulation of immune responses due to their anti-inflammatory capabilities and modulation of the cytokine expression at the site of infection (4). The release of exosomes from cells infected with intracellular pathogens such as *Toxoplasma gondii* and *Mycobacterium tuberculosis*. Furthermore, vaccinated mice with exosomes, containing mycobacterial antigens, have been involved in activation of $CD4^+$ and $CD8^+$ T-cells (5). GP63 is present in exosomes released by *Leishmania* spp. using mass spectrometry (MS) (6).

The aim of this study was investigating of some major proteases including GP63, other metalloproteinases, serine proteinases and cathepsins belonged to promastigote derived exosomes and its crude lysate using zymography technique.

Materials and Methods

Leishmania major promastigote culture and lysate preparation

The *L. major* promastigotes were cultured in RPMI 1640 supplemented with 10% of fetal calf serum and 1% of penicillin/streptomycin for six days at 25 ºC. Lysate of the promastigotes was prepared via repeated freeze-thaws in liquid nitrogen for five cycles followed by sonication and centrifugation at 3000 rpm for 10 min at 4 ºC. Then, supernatant was collected as lysate and protein concentration was assessed using Bradford method and bio photometer (Eppendorf, Germany).

Purification of exosomes

Approximately, 6×10^7 live *L. major* promastigotes in stationary phase were cultured in RPMI, containing 10% of FCS, at 25 ºC and then incubated in serum-free media for 22 h. Cell culture supernatant was collected for the isolation of exosomes using commercial exosome isolation kit (Exocib, Iran). This was centrifuged at 3000 rpm for 10 min to remove particles and debris. Samples were filtered through 0.22-µm filters. Then, 5 ml of the sample were added to 1 ml of Reagent A, mixed well and incubated overnight at 4 ºC. The mixture was centrifuged at 3000 rpm for 40 min at 4 ºC and supernatant was discarded thoroughly. Pellet of exosomes was resuspended in 100 µl of Reagent B and stored at -80 ºC until use.

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

Protein quantification was carried out using Bradford method and bio photometer. Electrophoresis was carried out as described by Lamelli (7) using 12% tris-glycine/sodium dodecyl-sulfate (SDS PAGE) gels and running buffer under non-reducing conditions. Separation of the *Leishmania* promastigote lysate proteins was carried out using mini gel electrophoresis system (BioRad, USA). Then, 20 µl of the sample, including 1 mg of the *Leishmania* promastigote exosome and lysate proteins per ml, were mixed with 20 µl of sample buffer with no reducing reagents. This was loaded and electrophoresed on gels at 80 V for 90 min. Coomassie blue stain was used to visualize protein bands (8).

Zymography

Proteases were separated using polyacrylamide gel (including 0.1% gelatin) electrophoresis and Laemmli buffer system. Then, 20 µl of the sample, including 1 mg of the *Leishmania* promastigote exosome and lysate proteins per ml, were mixed with 20 µl of sample buffer with no reducing reagents and loaded. For the detection of metalloproteinase, the gel slab was incubated in 2% triton X100 for 30 min and then in 50 mM tris-HCl buffer (pH 7.5) with 5 mM of $CaCl_2$ for 18 h at 37 °C. For the detection of serine proteases, incubation buffer included 100 mM tris-HCl (pH 8) with 10 mM of EDTA. For cysteine proteases, 0.1 M citrate buffer (pH 4) was used. Then, gels were stained with Coomassie blue R-250 and destained by soaking in 10% acetic acid, 50% methanol and 40% H_2O for at least 2 h (9).

Results

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

Proteins derived from the promastigote exosomes and lysate of *L. major* were analyzed using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). Bands with various molecular weights (MW) in kDa from *Leishmania* promastigote exosomes are shown in Lane 1, while further bands, belonging to MW marker and promastigote lysates, are demonstrated in Lanes 2 and 3, respectively.

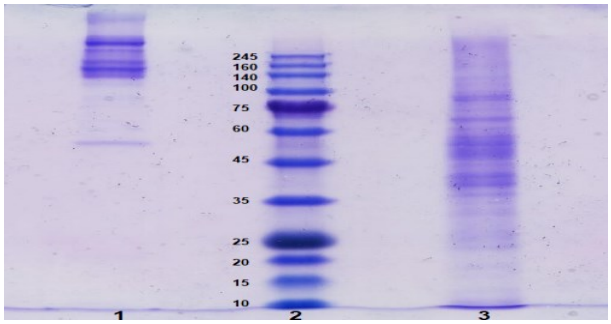

Fig. 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins from the promastigote lysate and exosomes of L. major

Metalloproteinase zymography

Zymography of the metalloproteinases from the promastigote exosomes included transparent bands with MW of approximately 130, 85, 77, 63, 50 and 48 kDa. One band included MW of approximately 63 kDa as the major surface protease (GP63) (Fig. 2). The highlighted bands were not detected in the *Leishmania* lysate; however, a transparent band with MW of approximately 47 kDa was seen in the *Leishmania* lysate.

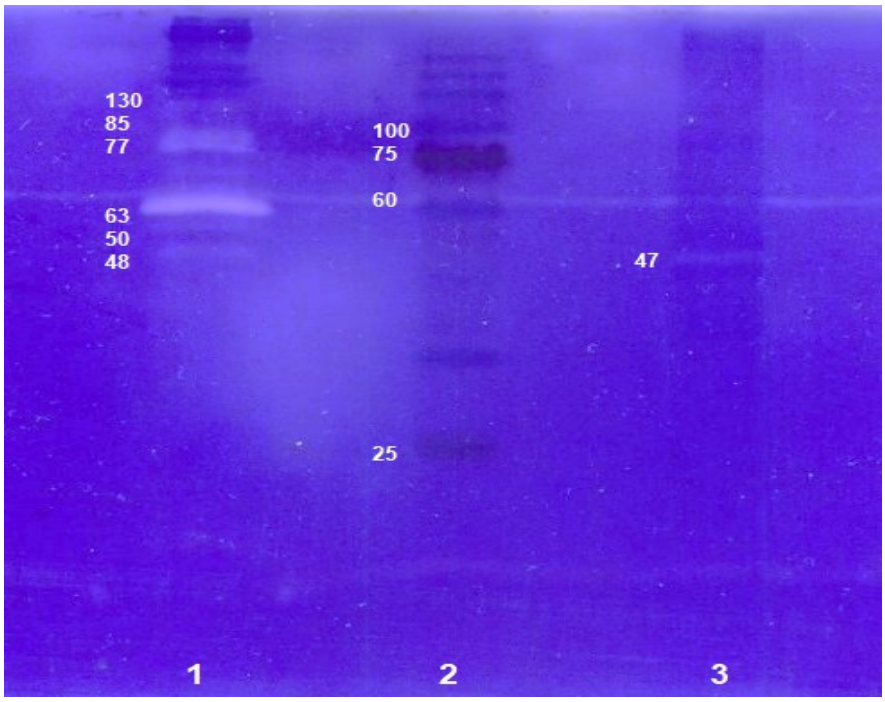

Fig. 2: Zymography analysis of the metalloproteinases from promastigote exosomes (Lane 1) and lysates (Lane 3) of L. major, including molecular weight marker (Lane 2). Gel was incubated in 100 mM tris (pH 7.5) containing 5 mM of CaCl2

Cathepsin zymography

Zymography analysis of the cathepsin from the promastigote lysate (Lane 2) showed transparent bands; of which, one band included MW of approximately 51 kDa and another one included MW of approximately 75 kDa as proteases (Fig.3). The bands were not investigated in the *Leishmania* exosomes (Lane 1).

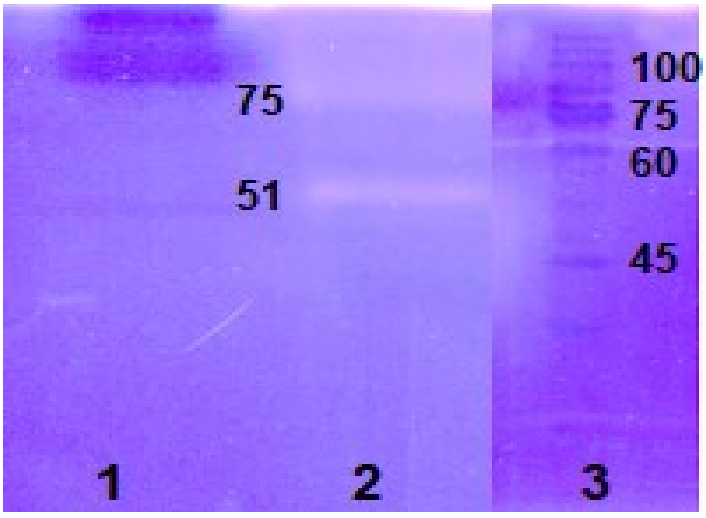

Fig. 3: Zymography analysis of the cathepsin from the promastigote exosomes (Lane 1) and lysates (Lane 2) of L. major, including molecular weight marker (Lane 3). Gel was incubated in 100 mM citrate buffer (pH 4)

Serin protease zymography

Zymography of the serin protease from the promastigote exosomes is shown in Fig. 4. Two transparent bands with approximately 48 and 65 kDa were seen, which belonged to serin proteinases (Fig. 2).

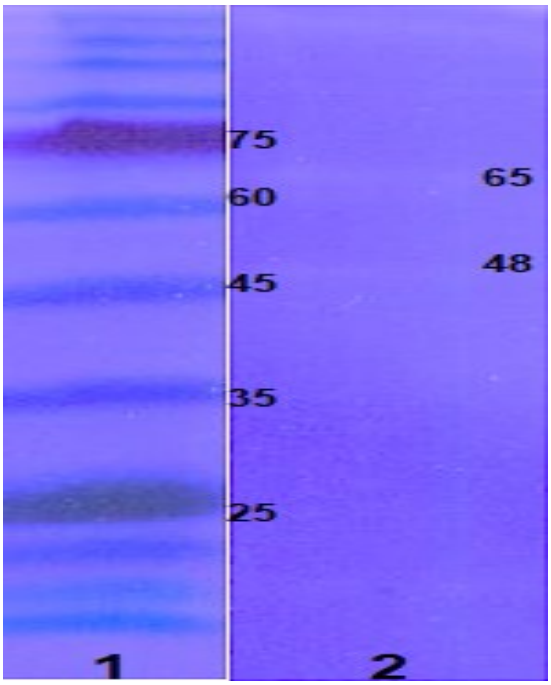

Fig. 4: Zymography analysis of the serin protease from the promastigote exosomes (Lane 2), including molecular weight marker (Lane 1). Gel was incubated in 100 mM tris-HCl (pH 8) containing 10 mM of EDTA

Discussion

Proteinases such as serine proteinases, cysteine proteinases and metalloproteases are virulence factors that enhance parasite survival and immune modulation in hosts. These enzymes play important roles in tissue invasion of the parasites by degradation of extracellular molecules, which can be addressed as appropriate candidates for the novel immunotherapy and chemotherapy of various parasite diseases such as leishmaniasis (2). Proteomic analysis of exosomes derived from the promastigotes of *L. infantum* showed 50 virulence factors, including GP63 and other proteases (10). Thus, the aim of this study was detection of proteases in *Leishmania* promastigote exosomes for the preparation of antigen fractions. In the present study, metalloproteinases, serin proteinases and cathepsins were investigated using zymography. Metalloproteinase zymography of the exosomes derived from *Leishmania* promastigotes showed transparent bands with MWs of approximately 130, 85, 77, 63, 50 and 48 kDa. The band with MW of 63 kDa could be linked to GP63 as a major surface metalloproteinase. These bands were not detected in *Leishmania* lysates. These results have shown that Gp63 is further concentrated in the exosome fraction and could be used as an appropriate antigen for serological diagnostic assessments. These results are similar to another study (10). GP63 is the most abundant protease in *Leishmania* promastigotes (11). This enzyme is majorly located in flagellar pockets of the parasite, attached to the surface of promastigotes via a GPI anchor. The enzyme seems to release directly from the para-

site. Shedding of GP63 to extra cellular space occurs with cleavage of GPI anchor by phospholipase C (12).

Technically, GP63 is a zinc-dependent metalloprotease, which its mRNA expression differentially occurs in various life stages of the parasite. In fact, GP63 includes important roles in complement processing and fixation for the protection of *Leishmania* spp. in their hosts (13). Furthermore, down regulation of GP63 expression has been demonstrated in the parasites, making them further susceptible to complement mediated lysis (13). GP63 can protect the parasite against antimicrobial peptides (14). In addition, GP63 can facilitate propagation of the parasite. Degradation of fibronectin by GP63 can protect *Leishmania* spp. from macrophages. Effects of GP63 on mitogen activated protein kinase (MAPK) signaling modulate cytokines of the immune responses (15). Moreover, the molecule can play roles in signaling cascades, affecting transcription factors such as cleavage of NF-*κ*B (16). As previously stated, GP63 has been demonstrated in *Leishmania* promastigote exosomes as a metalloprotease. Localization of GP63 on exosomes is carried out within the endosomes via transmembrane domains such as MT1-MMP. Through another mechanism, the protein binds to the exosome surface after secretion of its soluble form into the extracellular space. However, proteins on the surface of exosomes include similar orientation with their cell origin (17).

In this study, zymography of the serin protease from promastigote exosomes showed two transparent bands with MWs of approximately 48 and 65 kDa. Serine proteinases was reported as virulence factors and it was described their effects on patients' immune responses infected with *Leishmania* spp. (2). Serine proteinases protect the parasite in the midgut of insect vectors against mosquito enzymes as well as in the phagolysosomes of mammalian macrophages. Reports have indicated that the levels of serin proteases decrease in attenuated strains of *L. donovani* and it seems that a 115-kDa serine proteinase is linked to the parasite ability to infect its hosts (18). Subtilisin protease includes important roles in vertebrate-inhabiting stages of the parasite (19). The parasite serin proteinases play important roles in host immunomodulation and infection fate. Furthermore, host proteinases affect development of the lesions. In this study, two serin proteases with MWs of approximately 48 and 65 kDa were identified in promastigote exosomes (2). Cathepsin L-like proteases and their various expression have been detected in various stages of the parasite. Cysteine proteases play critical roles in metabolic routes, cell differentiation, passing through the tissue barriers, hemoglobin degradation, immune response modulation and apoptosis induction (20). Effectiveness of cathepsin L-like protease as a target for molecular diagnosis has been assessed (21). As a phylogenetic marker, it can help understand intraspecies variations and evolution of *L. infantum* (10). Antisense mRNA inhibition of cathepsin B gene has played important roles in parasite survival within the host macrophages by activating latent TGF-β1. (22).

In the current study, two transparent bands have been detected in zymography gels, belonging to cathepsin with MWs of approximately 51 and 75 kDa in promastigote lysates. However, no transparent bands have been reported for cathepsin in *Leishmania* promastigote exosomes. It seems that further methods are necessary for the final verification of these proteases.

Conclusion

Several metalloproteases, serin proteases and cathepsins have been isolated from exosomes and lysate of *L. major* promastigotes. In general, the 63-KDa glycoprotein (GP63) has been reported as the dominant surface metalloproteinase in this study, which plays im-

portant roles as a virulence factor in *Leishmania* promastigotes.

Acknowledgments

This study was supported by the Research Council of Faculty of Veterinary Medicine, University of Tehran. The authors thank Rastegar Reference Laboratory staff.

Conflict of interest

The authors declare that there is no conflict of interest.

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