In Vitro Effect of Proteinase from *C.Albicans* on *L.Donovani*

Azhar A.F. Al-Attraqhchi *PhD*, **Huda Th.Al-Marsome** *PhD*.

Abstract

Background: Visceral leishmaniasis is a sever and often fatal disease caused by *Leishmania donovani* .Therapeutic failures have been reported either due to initial failure to respond to treatment or relapse. The extracellular proteolytic activity is one of the several hydrolytic activities described for *Candida albicans*. Acidic proteinase is an important virulent factor which improved it's antibacterial activity. From this study we found that this enzyme has an anti leishmanial *in vitro*.

Objective: To detect anti-leishmanial effect of proteinase enzyme produce by *Candida albicans.*

Method: *C.albicans* standard strain (ATCC 10230) was cultured on wheat bran mediun PH 8.5 to produce proteinase enzyme in shaker incubater at 37^0C. The enzyme activity was determined by using 1% hemoglobin, pH 2.0, as a substrate[16] .The enzyme was precipitated with 0.5% and 50-75% ammonium sulphate consecutively. *Leishmania donovani* (MHOM/ IQ/82/BRC1) was used. Promastigotes were cultivated in RPMI medium, the number of parasites was adjusted to $1x10^6$cell/ml. The crude enzyme (1ml) was added at different concentrations.

Results: Intact proteinase and dilution of 1:2 were found to be effective on the growth of *leishmania* strain.

Conclusion: Candidal proteinase enzyme have an anti leishmanial activity (promastigote stage) and this may be considered as a good clue for the probability of using this enzyme as treatment agent for this parasite after trying it's activity on animal models.

Key words: Candidal proteinase, *L.donovani*.

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Introduction

Leishmania is a genus of the family trypanosomatide which are flagellated protozoa transmitted exclusively to man by the bite of female sandfly[(1)]. The life cycle of the genus *Leishmania* is divided in two stages [(2)].

1-Amastigote: An intracellular form of the parasite in the macrophages of the vertebrate.

2-Promastigote: It is motile form, found in the alimentary tracts of certain sandfly & in culture.

Infection of man by these protozoan results in a number of diseases called leishmaniasis. The visceral leishmaniasis is sever & often fatal disease that is caused by the *L. donovani* complex [(3)]. In Iraq, many investigators have noticed that children with visceral leishmaniasis showed marked differences in the severity of diseases as well as their response to chemotherapy [(4, 5, 6,7)].

It is remarkable that as the only recognized treatment for leishmaniasis is a heavy metal. In 1940, organic antimonial compounds were developed as a treatment and these remain the consensus treatment of choice for all forms of leishmaniasis. The toxic side effects of all these drugs drew the attention to search for a new safe or less toxic drug [(8)].

The extarcellular proteolytic activity is one of the several hydrolytic enzyme activities described for *C.albicans* [(9)]. Aspartyle proteinase is one of these enzymes; it plays an important role as a virulent factor [(10)]. This enzyme is secreted by pathogenic species of *candida* in *vivo* during infection [(11, 12)]. The enzyme is secreted

Dept. Microbiology, College of Medicine, Al-Nahrain University.
Adress Correspondence to:Dr. Azhar A.F. Al-Attraqhchi.
E. mail: tariq_963@yahoo.com.
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in vitro when the organism is cultured in the presence of exogenous proteins, usually bovine serum albumin, as the nitrogen source [13]. Proteinase production is believed to enhance the ability of the organism to colonize and penetrate host tissue and evade the host immune system [14].

The antibacterial activity of proteinase was determined against different types of bacteria isolated from patients and healthy individuals. The most sensitive bacteria were *Lactobacillus* spp. While *Pseudomonas aeruginosa* is the most resistant[15].Thus this study was conducted to evaluate the effect of proteinase enzyme on promastigote stage of *L. donovani in vitro* and its possible future to be used as an anti-leishmanial drug.

Materials and methods

Candida albicans strain:

The standard strain of *Candida albicans* (ATCC 10230) was cultured on Sabouraud' s agar, and then inoculated in 100 ml Sabouraud' s broth, supplied by the Biotechnology and Molecular Biology Department/ Baghdad University/Iraq.The culture was incubated in a shaker incubator at 37^0c for 24 hrs.

Two Erlynmyer's flasks of 250 ml containing 50 ml of wheat bran medium, PH 8.5, were inoculated with 1 ml of *C. albicans* broth culture. The flasks were incubated for 72 hours at 37^0c in a shaker incubator [16] .The cultures were harvested with 100 ml of 0.1M KH_2PO_4(pH 7.2) and centrifuged at 1500Xg for 30 minutes . The volume of the supernatant was measured and the enzyme activity was determined by using 1% hemoglobin (Oxoid), pH2.0, as a substrate.

Determination of proteinase enzyme activity:

The activity was determind according to Murachi (1970) [17] as follows:

Two glass tubes contained 1.9 ml of 1% hemoglobin, pH 2.0, and the other with 1.9 ml of 1% hemoglobin, pH 2.0. 0.1 ml of the sample (proteinase) was added to one of the tubes. Both tubes were incubated in a water bath at 35^0C for 10 minutes.

Three ml of trichloroacetic acid (TCA) were added to both tubes. 0.1 ml of proteinase was added to the control tube. Both tubes were centrifuged at 1500Xg for 15 minutes. The activity (unit/ml) of the supernatant was measured by a spectrophotometer at 280 nm.

Precipitation of proteinase with 0.5% ammonium sulphate

Ammoniun sulphate (29.11gm / 100ml) was added to the crude suspension gradually with stirring. The suspension was centrifuged at 300Xg for 15 minutes. The precipitate was dissolved in 5 ml of 0.1 M of acetate buffer at 4^oC, pH 5.0. Enzyme activity was measured for both supernatant and the dissolved precipitate as described previously. The protein concentration was determined according to the Biuret method [18].

Precipitation of proteinase enzyme with 50- 75 % ammonium sulphate:

Ammoniun sulphate (29.11 gm/ 100ml) was added gradually to the supernatant of the crude suspension with stirring [17]. The suspension was centrifuged at 3000Xg for 15 minutes and the precipitate was dissolved in 5 ml of 0.1M acetate buffer, pH 5.0[15]. The suspension was dialyzed against 2 liters acetate buffer of 0.1 M, pH5.0 at 4^oC over night. A further dialysis was done using the same conditions [15]. The activity of the enzyme and the protein concentration were determined.

The parasite:

Leishmania donovani (MHOM/ IQ/82/BRC1) was used. The parasite was stored in liquid nitrogen and maintained by continuous passage in Balb/ C mice. Promastigotes were

cultivated in RPMI medium [10]. On the day of the experiment, when the promastigotes were at the logarithmic growth phase, they were adjusted to 1x 10^6 cells/ ml in RPMI, supplemented with 10%fetal calf serum (FCS).

Treatment with the enzyme:

The crude enzyme (1ml) was added to the promastigote suspention (1 ml to each) at different concentrations (3 tubes for each concentration) (whole, diluted 1:2 with distilled water, 1:3, and 1:7). The total promastigotes count was determined each day for six days.

Results

Table 1 shows the results of the partial purification process of proteinase enzyme.

Table 1: Partial purification of proteinase enzyme from *C.albicans*.

Step	Volume(ml)	Activity (unit/ ml)	Protein (mg/ml)
Crude	50	100	5
Precipitation by 50-75% Ammonium sulphate	13	215	3

Table 2 shows the number of *L.donovani* promastigotes after the addition of proteinase. The enzyme had a lethal effect on the parasite in the concentrated, and that of the dilution of (1:2). The enzyme had no effect on the parasite when it was diluted further.

Table 2: Number of *L. donovani* promastigotes after the addition of proteinase enzyme from *C.albicans*.

Proteinase concentration	Cell con. Day 1	Cell con. Day 2	Cell con. Day 3	Cell.con. Day 4	Cell con. Day 5	Cell con. Day 6
Control	$5.0x10^6$ $4.5x10^6$ $5.5x10^6$ $5.0x10^6$	$13.0x10^6$ $12.0x10^6$ $13.5x10^6$ $12.7x10^6$	$20x10^6$ $21.0x10^6$ $20.0x10^6$ $20.3x10^6$	$27.0x10^6$ $28.0x10^6$ $27.5x10^6$ $27.3x10^6$	$35.0x10^6$ $36.0x10^6$ $35.0x10^6$ $35.5x10_6$	$50.0x10^6$ $60x10^6$ $55.0x10^6$ $55.0x10^6$
1:2	$3.0x10^6$ $3.5x10^6$ $5.0x10^6$ $3.8x10^6$	$7.0x10^6$ $7.5x10^6$ $9.0x10^6$ $7.8x10^6$	$7.5x10^6$ $10.0x10^6$ $6.0x10^6$ $7.8x10^6$	$3.0x10^6$ $3.5x10^6$ $4.0x10^6$ 3.5x10	zero	zero
1:3	$3.5x10^6$ $3.4x10^6$ $3.2x10^6$ $3.4x10^6$	$8.5x10^6$ $8.1x10^6$ $8.6x10^6$ $8.4x10^6$	$10.0x10^6$ $10.5x10^6$ $11.0x10^6$ $10.3x10^6$	$18.0x10^6$ $17.5x10^6$ $19.0x10^6$ $18.2x10^6$	$25.0x10^6$ $24.0x10^6$ $24.0x10^6$ $24.3x10^6$	$40.0x10^6$ $40.5x10^6$ $41.0x10^6$ $40.3x10^6$
1:7	$3.0x10^6$ $4.0x10^6$ $6.0x10^6$ $4.3x10^6$	$10.0x10^6$ $9.5x10^6$ $9.0x10^6$ $9.5x10^6$	$15.0x10^6$ $15.5x10^6$ $16.0x10^6$ $15.5x10^6$	$15.0x10^6$ $16.5x10^6$ $15.0x10^6$ $15.5x10^6$	$30.0x10^6$ $31.0x10^6$ $29.0x10^6$ $30.0x10^6$	$43.0x10^6$ $43.5x10^6$ $44.0x10^6$ $43.5x10^6$

Discussion

The pentavalent antimonials, amphotericin and pentamidine are used for the treatment of leishmaniasis. Some of these agents are not effective (especially for the most virulent strains) and all have serious toxic side effects, including cardiac and /or renal failure [8, 19]. Moreover, therapeutic failures have been reported in up to 15%of cases, which are either due to initial failure to respond to treatment or relapse [20]. A new oral treatment for visceral leishmaniasis(miltefosine)[21] has been established which had an overall cure rate greater than 90%. It was also found that it is specific and on the whole not too toxic. The major drawback is its effect on the fetus, and for this reason it cannot be used as a mass outpatient treatment [8], therefore we tried to find a new model for treatment , by using an anti-leishmanial agent such as proteinase enzyme.

From this study we concluded that candidal proteinase, have an anti-leishmanial activity (on the promastigote stage) .The best concentration used for such purpose was 1:1. We found there were no such previous studies done to compare with, except the antibacterial activity of this enzyme which determined against different types of bacteria isolated from patients and healthy individuals.

The effect of this enzyme may comes from that proteinases may attack the cell wall or the cell membrane or the inhibition or destruction of the parasite enzymatic activities.

To confirm these results much more studies are recommended to improve the mechanism(s) of activity, and to know this activity *in vivo* on the amastigote stage, and finally to assess the enzyme activity as a drug of choice against visceral leishmaniasis in particularly when systemic treatment of this disease is not effective and is too toxic (as mentioned in the introduction).

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