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BIOCHEMICAL CHARACTERIZATION OF DIPEPTIDYLCARBOXYPEPTIDASE OF *Leishmania donovani*

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Abstract

The incidence of parasitic infection, leishmaniasis, has been steadily increasing worldwide. Since, the existing chemotherapy of these diseases suffers from lack of safe and effective drugs and/or the presence of widespread drug resistance, there is an urgent need for development of potent, mechanism-based anti-parasitic agents. The peptidases of protozoan parasites are becoming increasingly important for their role in parasite survival and pathogenecity. *Leishmania donovani* dipeptidylcarboxypeptidsae (LdDCP), an angiotensin converting enzyme (ACE) related metallopeptidase has been identified and characterized as a putative drug target for antileishmanial chemotherapy. The kinetic parameters for LdDCP with substrate, Hip-His-Leu were determined as, Km, 4 mM and Vmax, 1.173 µmole/ml/min. The enzyme was more sensitive to 1,10 phenanthroline than EDTA and was 80% inhibited in presence of NaCl. Among various protease inhibitors, pepstatin was found as potent inhibitor of LdDCP.

Key words: Leishmaniasis, dipeptidylcarboxypeptidase, monovalent and divalent cations, chelating agents, protease inhibitors, Angiotensin converting enzyme, drug target.

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Abbreviations: DCP: dipeptidylcarboxypeptidase; **LdDCP**: *Leishmania donovani* dipeptidylcarboxypeptidsae; **EcDCP**: *Escherichia coli* dipeptidycarboxypeptidase; **ACE**: angiotensin converting enzyme; **HHL**: N-benzoyal-L-glycyl-L- histidyl-L-leucine.

INTRODUCTION

Leishmania parasites cause a wide spectrum of human and animal infections ranging from the life threatening visceral disease to the disfiguring mucosal and cutaneous forms of the disease. The disease is endemic in 88 countries of five continents (Africa, Asia, Europe, North America and South America) with a total of 350 million people at risk (1). These neglected diseases continue to pose a major threat to human health and economic development worldwide (7). Vaccines against leishmaniasis are still under development (10,16). Therefore, control of leishmaniasis relies mainly on chemotherapy which is far from ideal because of high costs, high toxicity and long term treatment requirements (6,14). Increasing incidences of therapeutic failures (13,20) and emergence of drug resistant parasites (8,19), now warrant an urgent need for new improved drugs.

The parasite leads digenetic life cycle with an extracellular promastigote stage in sand fly vector and an intracellular amastigote stage

occurring within mammalian macrophages (15). It is the amastigote form, which is responsible for pathogenicity due to its ability to encounter the hostile environment of the phagolysosome (10). It is proposed that parasite expresses a range of developmentally regulated genes presumably with a critical role in parasite survival in intracellular environment where chemical intervention may prove a possible route to control the infection. One such putative target is *L. donovani* dipeptidylcarboxypeptidase (LdDCP), an angiotensin converting enzyme (ACE) related metallopeptidase. It has been identified for the first time in any kinetoplast protozoan and found upregulated in amastigotes, the mammalian stage of the parasite (9). LdDCP belongs to M3 family of mono zinc peptidases which cleaves N-benzoyal-L-glycyl-L- histidyl-L-leucine (HHL), a substrate for ACE to release hippuric acid. Due to broader substrate specificity than aminopeptidase or oligopeptidase B, LdDCP may help in processing of small peptides released by a variety of endopeptidases during the life cycle of the parasite. Thus, LdDCP may have a role in parasite stage differentiation and is expected to play an indirect role in nutrition and pathogenesis. Further, Captopril, a known ACE inhibitor, was able to inhibit both LdDCP enzyme activity and *in vitro* parasite multiplication. These observations clearly indicated the therapeutic potential of parasite enzyme inhibition (9).

Recently, three dimensional model of LdDCP was generated based on crystal structure of *Escherichia coli* DCP (EcDCP) by means of comparative modelling and demonstrated several minor but potentially important structural differences at active site domain of LdDCP, EcDCP and ACE (2). Here, we present biochemical characterization of leishmanial dipeptidylcarboxypeptidase.

MATERIALS AND METHODS

pEXP5-CT/TOPO®TA expression vector, *Escherichia coli* TOP'10 , BL21 (DE3) pLysS strains were procured from Invitrogen Corporation USA. The protease inhibitors E-64, 1, 10 phenanthrolin, EDTA, trypsin inhibitor, pepstatin, PMSF, Angiotensin converting enzyme (ACE), N-benzoyal-L-glycyl-L- histidyl-L-leucine (Hip-His-Leu, HHL), NaCl, $MnCl_2$, $MgCl_2$, $CoCl_2$ and $CaCl_2$, $NiCl_2$, $CuCl_2$ and $ZnCl_2$, were obtained from Sigma Chemical Co., USA.

Recombinant leishmanial DCP was expressed in *E. coli* BL 21 pLys (DE3) expression host and purified to homogeneity as described earlier (9). Purity of the recombinant enzyme was checked on SDS-PAGE (12). Enzyme activity was measured according to the method of Cushman and Cheung, using N-benzoyl-L-glycyl-L-histidyl-L-leucine (HHL) a routine substrate used for angiotensin converting enzyme (ACE) (5). Activity of LdDCP was carried out spectrophotometrically, which is based on the direct measurement of released hippuric acid from HHL, which absorbs at 228nm.

Thermostability studies were performed by incubating purified LdDCP (~10 µg/ml) at different temperatures (−80^{0}C, −20^{0}C, 4^{0}C and 37^{0}C) in 30 mM Tris-Cl buffer, pH 7.5, in the presence of stabilizing agents (1 mg/ml BSA, 0.1%Tween 20 or 50% glycerol). At timed intervals, residual LdDCP activity was assayed using Hip-His-Leu as a substrate. Stability at 37^{0}C was followed up to 240 min. Stability at the other temperatures (-80^{0}C, -20^{0}C and 4^{0}C) was evaluated for up to 4 months. The resistance to freeze/thaw cycles was also examined. Change in enzyme activity as function of pH range (3.0-9.0) was also monitored.

Metal-ion-dependence was investigated by assaying LdDCP activities after pre-incubation of recombinant enzyme (~10µg/ml) with the metal chlorides (NaCl, $CaCl_2$, $MnCl_2$, $MgCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$ and $ZnCl_2$) (1mM) for 5min. rLdDCP residual activity was assayed using 1mM Hip-His-Leu in 30 mM Tris-HCl, pH 7.5 (11).

Effect of chelators and protease inhibitors namely, trypsin inhibiotor (both from bovine pancrease and soybean) and E64 was determined on activity of LdDCP. Different concentrations of inhibitors were added in reaction mixture prior to addition of enzyme. Enzyme activity was determined under standard assay conditions.

Protein concentration was determined by Bradford method using bovine serum albumin as standard (3).

RESULTS AND DISCUSSION

The peptidases of protozoan parasites are becoming increasingly important for their role in parasite survival and pathogenecity (18). Recently, we identified an ACE related dipeptidylcarboxypeptidase in *L. donovani* and demonstrated that this newly identified LdDCP could be developed as a drug target (9). It has also been shown that like ACE, the parasite enzyme cuts off C-terminal dipeptides from Hip-His-Leu (HHL) and releases hippuric acid. The Lineweaver-Burk plot with HHL as substrate gave a Km value of 4 mM for the LdDCP (2). To investigate the biochemical properties of the Leishmanial DCP, we expressed and purified the parasite recombinant enzyme as described earlier (9). It has been observed that recombinant LdDCP loses enzyme activity rapidly on storage at 4^{0}C or at -20^{0}C. In order to prevent loss of enzyme activity due to storage at different temperatures, the effect of three stabilizing agents (BSA, Tween 20 and glycerol) was studied. Incubation of purified LdDCP at RT without the addition of a stabilizer resulted in approximately

90% loss of the initial activity within 2 hrs. In the presence of 1 mg/ml BSA or 0.1% Tween-20, 50% of the initial activity was maintained up to 2hrs, while in presence of 50% glycerol, 95% of initial activity was maintained even after 4hrs (Fig 1a). Therefore, further conditions for stabilization of enzyme activity were standardized with glycerol only.

At 4^0C, without addition of glycerol there was complete loss of activity within 4 days. Addition of 5% and 10% glycerol resulted in some stabilization of the enzyme, as this preparation exhibited cleavage of HHL at the rate of 0.28mM/min/mg on day 4^{th} (96 hrs) (Fig.1b) which was approximately 30% of its initial activity. Interestingly, addition of 50% glycerol resulted in stabilization of enzyme activity to a significant extent. It exhibited specific activity of 1.06mM /min/mg which is about 70% of initial activity even after six days.

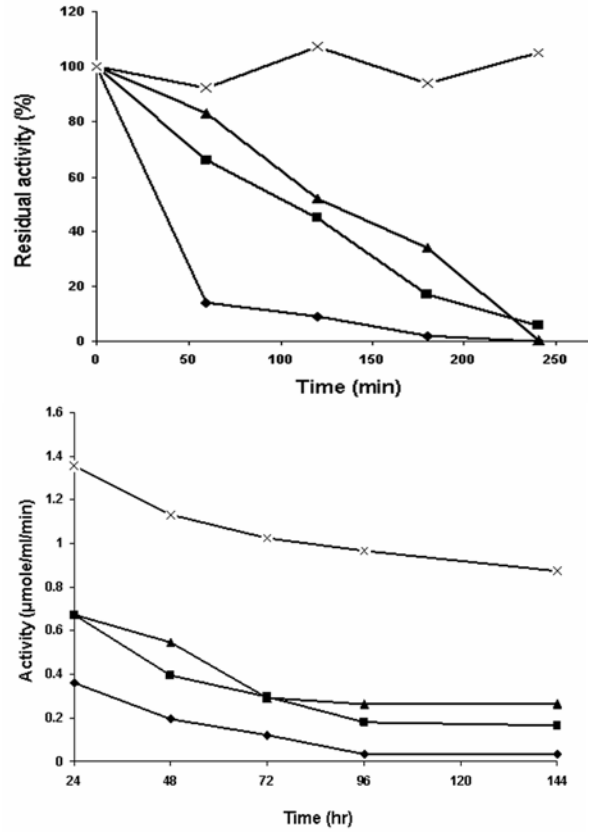

Figure 1. Effect of stabilizing agents on the activity of LdDCP at RT (♦) without stabilizing agent and (■) in presence of 1mg/ml BSA (▲) in presence of 0.1 % Tween-20 and (×) in presence of 50% glycerol. Each experimental data is a mean of at least duplicate observations.(b) The Effect of varying concentrations of glycerol on the activity of LdDCP: (♦) without glycerol (■) in presence of 5% glycerol (▲) in presence of 10% glycerol (×) in presence of 50% glycerol. Each point represents mean of at least duplicate observations.

Purified LdDCP was stable for at least 2 months in the presence of 50% glycerol at -20^0C and -80^0C. Glycerol not only stabilizes LdDCP but also stimulates its activity. In presence of 50% glycerol ~4 fold stimulation has been observed, when compared to activity without this stabilizing agent (data not shown). In absence of glycerol no residual LdDCP activity could be detected following two subsequent freeze/thaw cycles (gradual loss) (data not shown).

Further, to determine the pH optimum for LdDCP, activity assay was performed in presence of Tris-HCl buffer system (pH 3.0 to 9.0) using Hip-His-Leu as a substrate. Maximum activity of the LdDCP enzyme was observed between pH5.5 to pH7.5 (Fig.2)(20). The activity was markedly dropped below pH 4 and above pH 8.

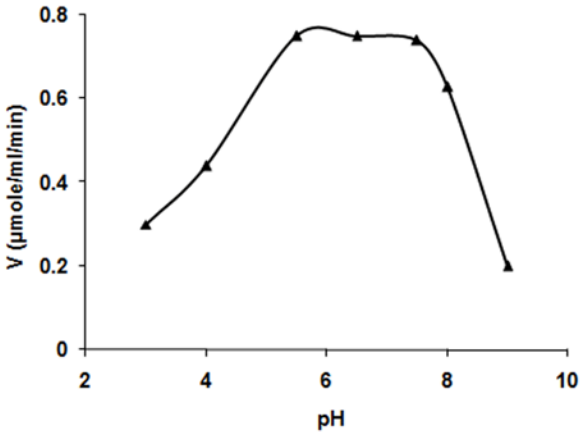

Figure 2. pH profile of LdDCP enzyme activity.

To examine the effect of ionic strength on the cleaving activity of LdDCP, 0, 20, 50, 100, 200 and 300 mM NaCl were incorporated in the standard reaction mixture using HHL as a substrate. The increase in NaCl concentration (0-300 mM) resulted in a loss of initial activity. In presence of 100 mM NaCl LdDCP cleaved HHL at the rate of 0.22 µm/ml/min which was only 20% of its initial activity (Fig. 3). Although the LdDCP and mammalian ACE share enzyme activity in terms of substrate specificity but significant differences were observed in their biochemical characteristics. Most important being inhibition in presence of monovalant cation NaCl. NaCl, known to stimulates human ACE at a concentration of 300 mM (4), had an inhibitory effect on both LdDCP and EcDCP (Fig.3 ; 11). High ionic strength decreases affinity of the substrate for the enzyme. It might be possible that salts ions electro- statically shield the charge

surface residues of LdDCP and does not allow the substrate for enzyme binding.

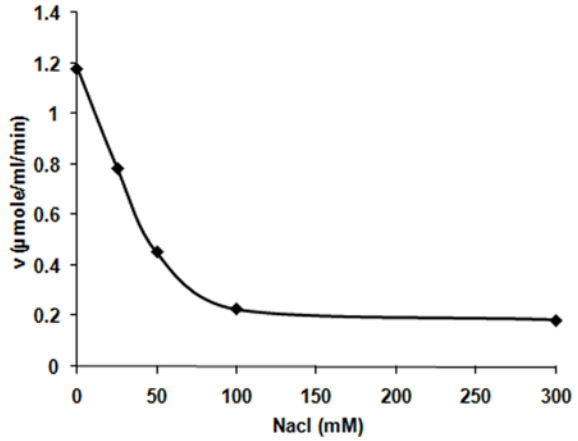

Figure 3. Effect of NaCl on the Hip-His-Leu cleaving activity of LdDCP. Assay was performed in presence of varying concentrations of NaCl (20-300mM) keeping the concentrations of substrate (1mM) and enzyme (~10µg/ml) constant.

To examine the effect of bivalent cations on the cleaving activity of LdDCP, 1mM of $CaCl_2$, $CoCl_2$, $NiCl_2$ and $CuCl_2$ were incorporated in the standard reaction mixture. Ca^{+2}, Mn^{+2}, Mg^{+2} and Co^{+2} stimulate LdDCP activity by 23-35 % while Cu^{+2} , Zn^{+2} and Ni^{+2} exhibited strong inhibition of LdDCP activity (Table1). Being Zn metallopeptidase, inhibition of LdDCP by Zn^{+2} may result from the formation of zinc monohydroxide [$(ZnOH)^{+}$] in the presence of elevated Zn^{+2} concentrations which might compete with the substrate by blocking the active centre of enzyme. In case of EcDCP, slight activation was also observed in presence of 1 mM Ca^{2+}. However, five-to eight fold stimulation was observed in presence of 50µM Co^{2+} (21). In accordance to LdDCP, Cu^{+2}, Zn^{+2} and Ni^{+2} strongly inhibited EcDCP also, reducing its activity to 16 and 9% of its normal level respectively (11).

In presence of 8 mM EDTA, LdDCP retained 37% of its maximum activity while only 1mM concentration of 1, 10 phenanthrolin was required to inhibit almost > 50% of its initial activity (Table1). Earlier studies (11) showed higher susceptibility of bacterial DCP to this chelating agent. 1, 10- Phenanthroline is a preferred chelator for Zn. In accordance, LdDCP exhibited much higher susceptibility to 1, 10 phenanthrolin than to EDTA, a general divalent ion chelator (Table 1). The catalytic site of Zinc dependent metallopeptidase consist of a Zn^{+2} bound by two histidine imidazoles and a glutamate (-COOH) in a distorted tetra hedral fashion. The data further confirm that DCP is a Zinc dependent metallopeptidase.

Table 1. Effect of Chelators, protease inhibitors and bivalent cations on LdDCP activity

Supplement	Concn(mM)	Relative LdDCP activity
None (control)[a]	-	1
Chelators		
1.10-phenanthroline	1	0.45
EDTA	8	0.37
Proteinase inhibitors		
Trypsine inhibitor[b]	4	0.40
E64	0.32	0.77
Pepstatin	0.002	0.48
PMSF	2.0	0.25
Bivalent cations		
Ca^{+2}	1	1.28
Mn^{+2}	1	1.35
Mg^{+2}	1	1.34
Co^{+2}	1	1.23
Cu^{+2}	1	0.15
Zn^{+2}	1	0.09
Ni^{+2}	1	0.08

[a]Control without inhibitor.
[b]from bovine pancreas.
[c]bivalent cations were added as Cl salts.

Protease inhibitors affected the LdDCP activity in a dose dependent manner. In presence of 320 µM concentration of E64 (N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]agma - tine), the irreversible inhibitor of cysteine proteases, 35% inhibition could be achieved. On the other hand, trypsin inhibitor bovine pancreas (BPTI) exhibited 60 % inhibition of LdDCP activity at 4 mM concentration (Table1) (11). BPTI is a monomeric polypeptide contains 58 amino acid residues and probably, the large conformation of BPTI might hinder its fitting into the active site. Pepstatin at 2 µM concentration inhibited 52% enzyme activity while PMSF at 2mM inhibit almost 75% of LdDCP activity. Taken together, the present study clearly demonstrates that although LdDCP and mammalian ACE share significant enzyme activity in terms of substrate and ACE inhibitors but significant differences were observed in their biochemical characteristics.

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Other articles in this theme issue include references (22-37).

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