Proteases as Virulence Factors in *Leishmania*: Focus on Serine Proteases as Possible Therapeutic Targets

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Abstract Leishmaniasis is one of the most assorted and intricate of all vector borne diseases caused by the genus *Leishmania*. Survival of *Leishmania* parasites inside the mammalian host needs a set of virulence factors, among them, *Leishmania* proteases have paramount importance. Several of these proteases have been identified as potential virulence factors for their crucial roles in the invasion of the host via parasite migration through tissue barriers, degradation of host proteins for nutrition purpose, immune evasion and activation of inflammation. Hence, the investigation on proteases in *Leishmania* is proposed as a valuable approach to enhance our knowledge on host-parasite interaction. Through various studies, a number of metalloproteases and cysteine proteases have been implicated as major components in host invasion by modulating host cell signaling for the establishment and continuation of infection by *Leishmania*. But, the roles of serine proteases in leishmaniasis have not been investigated adequately. In this review, we will discuss the significance of *Leishmania* proteases in parasite lifecycle and their possible accountability as a new drug target with special emphasis on *Leishmania* serine proteases.

Keywords Leishmaniasis • Matrix metalloproteases • Oligopeptidase B • Cysteine protease • Serine protease • Virulence • Protease inhibitor • Drug target • Chemotherapy

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1 Introduction

Leishmaniasis is caused by an obligate intracellular protozoan parasite of the genus *Leishmania*. The disease is manifested in different clinical forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) of which VL is the most severe form of leishmaniasis. According to World Health Organization (WHO), 350 million people are at risk of contracting one of the forms of the disease, 12 million cases in 88 endemic countries worldwide and also with an annual-incidence of 1.5 million new cases [1]. Currently, leishmaniasis is spreading due to co-infection with human immunodeficiency virus (HIV) [2].

All types of leishmaniasis are transmitted by the female phlebotomine sand flies. *Leishmania* parasites alternate between two distinct developmental stages. The motile flagellated promastigote forms multiply and develop extracellularly in the alimentary tract of the blood sucking female sand fly vectors and are transmitted during the blood meal into mammalian host. Inside the mammalian hosts promastigotes infect macrophages of the reticuloendothelial tissue and differentiate into nonmotile amastigotes forms which multiply as such in the phagolysosomal vacuoles.

Like other intracellular protozoan parasites, *Leishmania* survive inside the host macrophages (Mφs) by implying various mechanisms [3, 4] and allow them to evade and suppress the host immune system, thereby granting their survival in the host body. In order to establish themselves in the host, *Leishmania* promastigotes have to escape Mφ microbicidal action and amastigotes have to repress Mø killing abilities to re-invade new Mφs for persistent *Leishmania* infection [5, 6]. Following phagocytosis, *Leishmania* persuade the harsh environment through the inhibition of hydrolytic enzymes, toxic metabolic products, cell signaling, cytokine production and other events [7]. These tactics permit *Leishmania* to successfully undermine the host innate and acquired immune responses to promote their survival. The effective elimination of parasites by macrophages depends on the activation of appropriate immune responses [8, 9]. So, *Leishmania* have developed mechanisms to subvert the microbicidal activity of Mφs [10] where the host Mφs produce nitric oxide (NO) through the induction of iNOS, in response to extracellular signals, including IFN-γ and LPS [11] and this NO generation by activated Mφs is the prerequisite for intracellular killing of amastigotes. *Leishmania* enter into the host Mφs by receptor mediated endocytosis and avoid complement mediated lysis by cleaving C_{3b} to C_{3bi} [7]. Inside the phagolysosomes of the host Mφs, *Leishmania* promastigotes can also hinder phagosome–endosome fusion and protect themselves from toxic oxygen metabolites generated during the macrophage oxidative burst by scavenging hydroxyl radicals and superoxide anions [12]. *Leishmania* also escape microbicidal action of host Mφs by modulating host cell cytokines production, where induction of a Th1 type immune response is associated with clearance of *Leishmania* infection and a Th2 type immune response leads to persistence of infection. In *Leishmania* infection TGFβ, IL-10 and PGE2 have been implicated as important immunosuppressive signaling molecules and also inhibit the production of the Th1 response-promoting cytokines IL-1, IL-12 and TNF-α [13, 14].

Both promastigotes and amastigotes alter the signaling pathways of Mφs in order to block their killing functions [14–17]. PKC signaling is known to play a key role in the regulation of Mφ functions activating Th1 response leading to NO production and oxidative burst [14] but, restraining of PKC activation by *Leishmania* parasites impair subsequent signaling phenomena [14, 15].

Leishmania modify host signaling through the disruption of cellular phosphorylation [18] by expressing endogenous phosphatases that cause a decrease of macrophage PKC activity and inhibition of MKP1 (p38) and MMKP3/PP2 (ERK1/2) activation leading to the up regulation of IL-10 and down regulation of NO and TNF-α production [14]. Consequently, *Leishmania* mediated IFN-γ inducible tyrosine phosphorylation and activation of JAK1, JAK2, and STAT1 pathway [19] has been shown to involve the activation of the cellular protein tyrosine phosphatase SHP-1, responsible for dephosphorylating MAP kinases 1 and 2 [14, 15]. The *Leishmania* parasites can also decline the nuclear translocation of NF-κB in monocytes with an outcome of a decrease in IL-12 production [20].

Various virulence factors enable *Leishmania* to invade and establish infection inside the mammalian host [7, 14, 21–25], these factors include major surface protease (GP63), cysteine proteases (CPs), serine proteases (SPs), lipophosphoglycan (LPG), A2 protein family, glycosylinositol phospholipids (GIPLs), secreted acid phosphatases (SAPs) and kinetoplastid membrane protein 11 (KMP-11) etc. However, the specific roles of these molecular determinants are still under debate [26, 27]. Therefore, the elucidation of the mode of action of these virulence factors toward the host cell is of utmost importance to accomplish a more comprehensive view of the host-parasite interactions as well as immune modulation and thus also focus a new insight of target molecules for therapeutic intervention of leishmaniasis.

2 Proteases as Virulence Factors in *Leishmania*

Among the numerous parasitic virulence factors, parasite-derived proteases receive supreme importance due to their vital roles in the parasite life cycle and pathogenesis. Parasites produce a wide array of proteases which are essential for degradation of the tissue barriers for migration of parasites to specific sites, cleavage of host proteins for their essential nutrients, activation of inflammation that assure their survival and proliferation to sustain the infection [28–33]. A variety of *Leishmania* derived proteases have been shown clinically important for diagnosis and vaccine development. Several studies have illustrated metalloproteases, cysteine proteases, aspartic and recently serine proteases to be essential for *Leishmania* infection (Table 1).

The *Leishmania* genomes encode a large number of proteases [34]. *L. major* is expected to contain at least 154 peptidases (including aspartic-, cysteine-, metallo-, serine- and threonine-peptidases as well as one protease of unknown catalytic type) that represent around 1.8 % of the genome (Fig. 1) [34]. Comparative genomic analysis with the different species of the genus *Leishmania* have shown that the numbers of proteinase genes remain fixed among the various species (http://merops.sanger.ac.uk/).

Table 1 Biological roles proteases in leishmaniasis

Classes of proteases	Biological name	*Leishmania* species	Immunological functions
Metallo-proteases	GP63	*L. donovani* *L. amazonensis* *L. mexicana* *L. major*	Inactivates IgG by hydrolysis, evade complement-mediated lysis by cleaving of C3b into C3bi, degrades NF-κB and inhibit IL-12 and NO expression, protects against antimicrobial peptides, affects the natural killer (NK) cell functions, alters signaling by SHP-1 activation leading to inhibition of JAK2/STAT1α pathway, IRK1 kinases and MAPK, degrades JNK, hydrolyses MARCKS/MRP, cleaves mTOR, inactivates transcription factors by cleaving c-Jun and AP-1 [7, 14, 15, 18, 21, 22, 25, 37–40].
	Intracellular metalloprotease (MP-Ld)	*L. donovani*	MP-Ld located extensively near the flagellar pocket region, appears to play important roles in parasitic development [65]
Cysteine proteinases	CPA	*L. infantum*	Plays a role in infection of mammalian host cells [77]
	CPB	*L. mexicana* *L. major* *L. amazonensis* *L. chagasi* *L. pfanoi*	Induces Th2-associated immune response, induces lesions; inhibits of IL-12 production by degrading NF-κB, inhibits of NO generation by cleaving STAT-1 and AP-1 transcription factors, degrades of IκB-α, IκB-β transcription factors, prevents antigen presentation by degrading MHC class-II molecules, inhibits NK cells proliferation and cleaves CD4 glycoprotein of human T cells [36, 62, 67–69, 88, 95]
	CPC	*L. mexicana* *L. chagasi*	Subverts microbicidal effects of macrophages and contributes to resistance, induces TGF-β expression in human cell culture [36, 75, 90, 91]
Serine proteinases	Oligopeptidase B	*L. donovani* *L. mexicana* *L. major*	Help to differentiate from promastigote to amastigote. Regulate levels of enolase on the parasite cell surface and facilitate parasite entry into macrophages. Contributes to retain macrophages infection [111, 121, 193]
	Subtilisin type seine protease	*L. donovani*	Plays essential role in promastigotes to amastigotes differentiation, detoxifies reactive oxygen intermediates and maintains redox homeostasis, and is essential for *Leishmania* virulence [136]
	Serine protease	*L. amazonensis*	Enhances the *Leishmania* infection by promoting Th2-type of immune responses and is essential for parasite survival [138, 190]
	115 kDa Serine protease (pSP)	*L. donovani*	Associated with metacyclic promastigotes, located mainly at flagellar pocket region, confers significant protection via IFN-γ induced down-regulation of TNF-α mediated MMP-9 activity in experimental visceral leishmaniasis [140]
	Intracellular serine protease (SP-Ld)	*L. donovani*	Localized in the flagellar pocket as well as at the surface of the parasite, it down regulates the phagocytic activity of macrophages [65]
Aspartic proteases	Presenilin 1 (PS1) type	*L. major*	Cleaves type I membrane proteins and effectively involved in autophagy [66]
	Signal peptide peptidase (SPP) type		Cleaves the transmembrane domains of signal peptidases [66]
	Ddi1-like protein	*L. major* *L. mexicana*	Essential for *Leishmania* growth and macrophages infection [142, 146, 147]

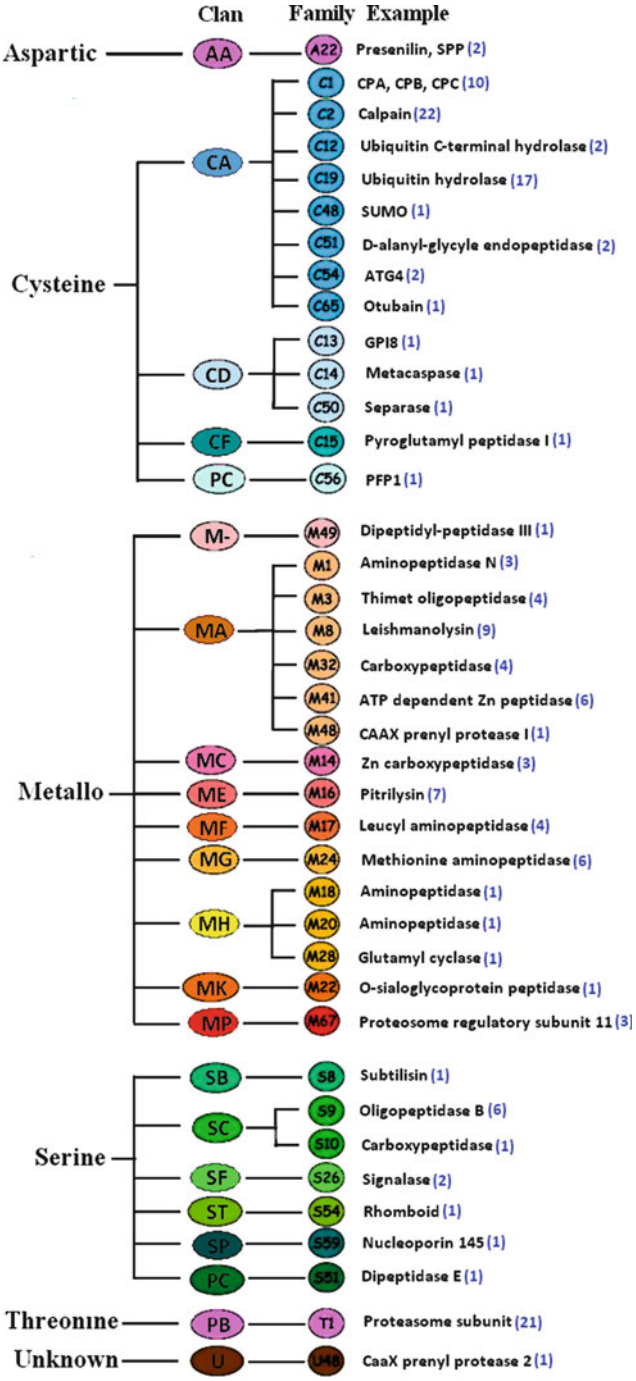

Fig. 1 Clans and families of *L. major* peptidases. Nomenclature is done on the basis of the MEROPS database (http://merops.sanger.ac.uk/). The estimated number of peptidases in each family is represented by *numbers* within *brackets* [34]

Many proteases are reported in both forms of *Leishmania* and their roles in the parasite physiology and immunoinvasion have been elucidated. A number of metalloproteases and cysteine proteases have been suggested to be virulence attributes that contribute to *Leishmania* pathogenesis by modulating the host cell signaling [25, 31–39]. Although, serine proteases have been extensively studied because of their imperative roles in parasite survival and pathogenicity [25, 39–44], roles of serine proteases in leishmaniasis have not been investigated adequately. In this context, the present review deals with the leishmanial proteases with special reference to the serine proteases, their possible involvement in *Leishmania* pathogenesis and to consider them as promising drug target for the prevention of the disease.

2.1 Metalloproteases

Promastigote major surface protease (MSP) leishmanolysin or GP63, the most abundant surface glycoprotein of *Leishmania*, belongs to the clan MA, M8 family of endopeptidases [34]. Its abundant expression of *Leishmania* promastigotes surface is well explored and its presence in amastigotes is also known [45]. It is bound to the surface membrane by a GPI (glycosyl phosphatidyl inositol) anchor which can be cleaved in vitro by phospholipase C (PLC) but its release *in vivo* depends on autoproteolysis [46]. *L. braziliensis* alone has at least ninety-seven metalloproteinase (http://tritrypdb.org, http://blast.ncbi.nlm. nih.gov/) and 16 families of metallopeptidases were identified in *L. major* (Fig. 1) [34]. GP63, accounting for about 1 % of the total protein in promastigotes of *Leishmania*, are potentially important during different stages of the life cycle [37]. GP63 share several characteristics with mammalian matrix metalloproteases (MMPs) that includes degradation of the extracellular matrix, cell surface localization, activation by Zn^{2+} and inhibition by several chelating agents and α-2-macroglobulin and like MMPs, it has also a wide range of substrates including casein, gelatin, albumin, haemoglobin, and fibrinogen [37, 47].

The zinc dependent metalloprotease GP63 can act in the sandfly midgut as well as macrophages parasitophorous vacuoles. In the amastigote, leishmanolysin (GP63) is located in the large lysosomes [48, 49]. At least 18 metalloproteinase genes were detected in *L. chagasi* and seven metalloproteinase genes have been identified in *L. major* [46, 50]. The up-regulation of GP63 expression in invasive metacyclic promastigotes suggests that the protease plays an important role in the early stages of infection of the mammalian host [51]. Examination of *Leishmania* strains expressing varying levels of GP63 has suggested that it participates in direct binding of the parasite to macrophages [22, 39]. Besides its presence in different species of *Leishmania*, GP63 has also been reported in various trypanosome species like *Crithidia fasciculata*, *T. brucei*, and *T. cruzi* [37]. More recently leishmanolysin homologs have been found in *Trichomonas vaginalis* as well [52].

GP63 plays important diverse roles in leishmanial pathogenesis. It helps *Leishmania* promastigotes to evade complement-mediated lysis (CML) by proteolytic cleavage of C3 complement into inactive iC3b (inactive C3b) thereby helping

the parasite to avoid complement pathway and thus enhances phagocytosis of promastigotes by host Mø through macrophage receptors CR3 [53]. It also favors promastigote migration through the extracellular matrix (ECM) by degrading the extracellular matrix components such as fibronectin and collagen IV and thereby further facilitates the parasite adherence to macrophages [54]. As an immunemodulator, GP63 diminishes both T cell responses either by cleaving CD4 molecules from T_h cells or also by degrading many other intracellular peptides presented by major histocompatibility complex class I (MHC-I) molecule [55, 56]. Additionally, GP63 mediated enzymatic degradation of antimicrobial peptides causes resistance of the parasite to apoptotic killing by these peptides [57].

GP63 modulates host negative regulatory mechanisms by degrading various kinases and transcription factors. It is responsible for the hydrolysis of the myristoylated alanine-rich C kinase substrate related protein (MRP), a major PKC substrate in macrophages and thereby inhibits PKC activation [58]. Recently it has been proposed that GP63 dependent alternative mechanism could be involved in PKC alteration [22]. It was also reported that *Leishmania* GP63 is able to rapidly reach the intracellular milieu of the host macrophage through lipid raft and activate host protein tyrosine phosphatases (PTPs) [18, 38]. It induces activation of the protein tyrosine phosphatase (PTP) SHP-1 through a lipid raft-based mechanism causing inhibition of JAK/STAT pathways, IFN-γ stimulation and reducing NO production [22, 59]. Thus the progression of leishmaniasis involving PTPs activation also requires the proteolytic mechanisms involving GP63.

Furthermore, GP63-mediated SHP-1 activation involves MAPK inactivation where JNK kinase and its downstream signaling target namely c-Jun, is cleaved by GP63 thus directly affecting MAPK activation [60]. At the same time, GP63 mediates cleavage of p65subunit of the NF-κB into a smaller subunit (p35) that enters the host cell nucleus and triggers the expression of chemokines [61]. Taken together, GP63 was found not only to degrade NF-κB completely but also implicated in the proteolysis of c-Jun, the central component of the transcriptional complex AP-1 leading to decreased IFN-γ-induced NO production [60, 62].

Natural killer (NK) cells play important roles in innate immunity via cytotoxic activity and early cytokine production against pathogens, including parasites. Proliferation, receptor expression and IFN-γ released by natural killer (NK) cells have been shown to be affected by *Leishmania* GP63 therefore inhibiting the Th1 type immune response with parasite infection and has also been shown to cleave mTOR to control translational system of host cells [63, 64]. Destabilizing the proper functioning of the transcriptional machinery by *Leishmania* GP63 results in the enlistment of the macrophages to serve as host, thereby precluding the expression of host factors such as IL-12 and iNOS that threaten their survival [22, 38, 39]

Interestingly, another metalloprotease (MP-Ld) was also identified in *L. donovani* promastigotes [65]. Both immunofluorescence and immune-gold electron microscopy studies revealed that MP-Ld is located extensively near the flagellar pocket region (Fig. 2). It seems to be of collagenase type as it degrades azocoll with maximum efficiency. It was predicted that MP-Ld played major and important role in parasitic development rather than in the infection process.

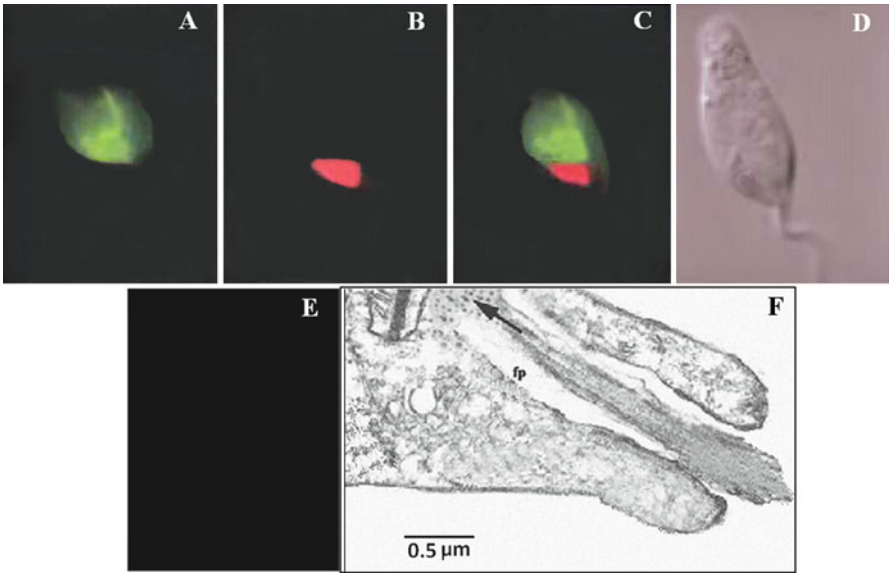

Fig. 2 Localization of *L. donovani* intracellular metallo protease (MP-Ld) by confocal immunofluorescence and immune-gold electron microscopy. MP-Ld was envisioned by FITC-labeling (**a**), TRITC conjugated con A was used to label the flagellar pocket (*red*) (**b**), Merged images showing co-localization of MP-Ld within the flagellar pocket (**c**) and (**d**) is the phase contrast image of MP-Ld. No fluorescence was detected in presence of corresponding pre-immune serum (**e**). *L. donovani* promastigotes showing the presence of gold particles (**f**) indicate distribution of MP-Ld near the flagellar pocket denoted by the *arrow* [65]

Overall view of these evidences suggest that metalloproteases of *Leishmania* are of significance that could unveil the molecular mechanisms of host-parasite interactions where GP63 is a profound virulent factor of *Leishmania* and could be an effective target of novel therapeutic and prophylactic approach for prevention of leishmaniasis.

2.2 Cysteine Proteases

Leishmania expresses many distinct genes (Fig. 1) encoding a total of 65 cysteine proteases and they are involved in a wide range of important biological processes [36]. *L. major* was found to have members of four clans of cysteine peptidases, consisting of enzymes of eight families of clan CA, three families of clan CD and one family each of clans CF and PC [66]. Cysteine proteases (CPs) have been demonstrated as important virulent factor as they are essential for *Leishmania* survival, replication, development, metabolism, host cell infection and evasion of host immune response [36, 67].

The proteases of the papain family (clan CA, family C1) are the most extensively studied proteases of *Leishmania*. The best characterized of these enzymes are the cathepsin L-like A (CPA) and B (CPB) families of cysteine proteases and the cathepsin B-like C (CPC) family of cysteine proteases, all of which are lysosomal in amastigote stages [66]. Many studies have identified CPs as prevalent virulence factors in *Leishmania* genus [36]. It has been investigated that both CPB and CPA facilitates effective autophagy and differentiation of the *Leishmania* [68]. The proteases have been demonstrated as potential drug targets and vaccine candidates [66, 69–71] as they are essential for the growth of *Leishmania* and for the progression of lesions.

Roles of these proteases in the modulation of host immune response have been reported. The lack of both CPA and CPB lead to increased production of Th1-type cytokines response, and reduced production of IL-4, a signature cytokine of the Th2-type immune response [72–74]. CPC has also been found to exacerbate the disease [75]. There is evidence that CPC can also play a relevant role as a key *Leishmania* virulence factor as it may contribute to some of the immunoregulatory activities of *L. chagasi* by inducing TGF-β expression [36, 75, 76]. Based on gene suppression studies CPA from *L. infantum* was found to be responsible for virulence of the parasite [77, 78].

Cathepsin L-like proteases of *L. pifanoi* and *L. mexicana* and the cathepsin B-like protease of *L. major* localized in lysosomes implicates the involvement of these enzymes in protein degradation [36, 70, 79]. Cathepsin-L like cysteine protease (CPB) promotes a Th2-type of immune response by cleaving the IL-2 receptor CD25 and the low-affinity IgE receptor CD23 [73, 79, 80]. Moreover, active participation of leishmanial CPs in T-cell mediated immunity is due to the presence of T-cell epitope at the COOH-terminal region of the protease itself [81, 82]. In addition, computational analysis of *Leishmania* CPs also reveals that they contain potential epitopic regions [83]. Due to immunogenicity of CPs, they have been used as vaccine candidates with different degree of protection in animal model [84–87]

A high CP activity was observed in extract of *L. amazonensis* amastigotes, but promastigotes from the exponential or stationary phases exhibited very low proteolytic activity [88–90]. In several species of *Leishmania*, there is an association between the level of CP expression and virulence [72, 91, 92]. CP is highly expressed in amastigotes and very low level in metacyclic promastigotes which might specify its central roles for intracellular survival of the parasite [36]. Correspondingly, *Leishmania* lacking cysteine proteases or parasites treated with specific cysteine protease inhibitors massively exhibits less infectivity [92–94].

Leishmania CPs have also been implicated in the inhibition of the crucial role of host cysteine proteases in the procession of antigen presentation by degrading MHC class II molecules in the parasitophorous vacuole [95] and partially inhibit host immune response. *Leishmania* amastigote CPs may also involve positive alteration of PKC mediated signaling that causes an enhanced expression of MKP3, PP2 and MKP1favoring intracellular survival [16, 22]. Another important function of CPs as virulent factors is the degradation of the transcription factors STAT1 and AP-1 which subsequently hinder NO production in host macrophages [96]. Alternatively, *Leishmania* CPs are also capable to disrupt NF-kB signaling by drastic cleavage of

NF-κB family proteins with downregulation of IL-12 production and concomitant persistence of infection in host macrophages [62, 97].

Collectively, *Leishmania* CPs are considered as a key factor with potential attribute in disease pathogenesis and hence might be addressed for developing a suitable drug of leishmaniasis.

2.3 Serine Proteases

Serine proteases are extensively dispensed in nature i.e. in all cellular organisms and more than one third of all known proteolytic enzymes are serine proteases grouped into 13 clans and 40 families [98]. They are a diverse group of enzymes that are characterized by the presence of three critical amino acids-histidine, aspartate, and serine-in the catalytic site [99]. These residues form together the "catalytic triad" of serine proteases. The family name is originated from the nucleophilic 'Ser' in the enzyme's active site, which attacks the carbonyl moiety of the substrate peptide bond to form an acyl-enzyme intermediate thus to hydrolyze peptide bonds [100]. The "catalytic triad" is associated with many families of seine proteases including the trypsin, subtilisin, prolyl oligo peptidase and serine carboxypeptidase families [101].

Serine proteases can be classified into three groups based mainly on their primary substrate preference: (1) trypsin-like, (2) chymotrypsin-like and (3) elastase-like. Trypsin family proteases represent the most abundant group in vertebrates, where they function in blood coagulation, the complement cascade, intestinal digestion, in inflammatory responses, reproduction and many other physiologic processes as in development, maintenance, and pathology of the nervous system [102–105].

In general, serine proteases of protozoan parasites and some bacteria are of the subtilisin (SB) type and in many cases oligopeptidase B (OPB) type. Chymotrypsin, trypsin and elastase (trypsin family) share closely-similar structures containing active serine residue at the same position (Ser-195), while subtilisins have Ser residue at 221. Subtilisin (serine endopeptidase) is a non-specific protease.

In the trypanosomatids, serine protease research has generally centered on the oligopeptidase B (OPB) and prolyl oligopeptidase (POP) [41, 106]. During entry into the host cell, it is supposed that *Trypanosoma cruzi* OPB augments host cell penetration by eliciting Ca^{2+}-signaling mechanism [42, 107]. *T. cruzi* prolyl oligopeptidases (POP) may be important to degrade extracellular matrix proteins such as collagen and fibronectin to facilitate parasite invasion process [108] as the penetration of *T. cruzi* into host is reduced in the presence of selective exogenous OPB and POP inhibitors [32, 109–111].

Serine proteases in apicomplexans have mainly centered on protein processing and other functions related to intracellular survival [112]. To date, many serine proteases have been found to be essential virulence factors in protozoan parasites including *Plasmodium falciparum*, *Eimeria tenella*, *Toxoplasma gondii*, *Babesia divergens*, *Perkinsus marinus* etc. [44, 112–118]. *Helminthes* and *Schistosomes* parasites exploit their serine proteases in anticoagulation and invasion respectively [32].

Excluding metallo and cysteine proteases, *Leishmania* also contain at least twenty-three serine proteinases (http://tritrypdb.org, http://blast.ncbi.nlm. nih.gov/). The activity of a serine peptidase was first purified and characterized from soluble extracts of *L. amazonensis* promastigotes [119]. This serine peptidase was characterized as an oligopeptidase as it can't hydrolyze proteins or large peptides, but it cleaves only small peptides substrates, at their carboxyl side. *Leishmania* OPB was subsequently described in *L. major* in 1999 [120]. By means of mass spectrometry and gene deletion approach, the *Leishmania* oligopeptidase B (OPB; Clan SC, family S9A), was identified and characterized [121]. The OPB activity was detected in both promastigote and amastigote stages of *Leishmania*. However, this activity was significantly elevated in the amastigote stage for both *L. donovani* and *L. Mexicana*. The *L. amazonensis* OPB was cloned and sequenced and was found to be 90 % identical to *L. major* and *L. infantum* OPB and 84 % identical to *L. braziliensis* [122]. It is important to keep in mind that *Trypanosoma* species do not express enzymes showing serine protease activities, but only serine oligopeptidases with specific functions in many steps of mammalian cell invasion [123, 124].

Furthermore, some serine proteases have been reported in *Leishmania* in secreted as well as in intracellular form [65, 125–131]. A detergent soluble 110 kDa serine protease [125] and a 68 kDa intracellular serine protease in aqueous extract were identified and characterized from *L. amazonensis* promastigotes [126, 132]. At the same time extracellular serine proteases from different species of *Leishmania* like *L. amazonensis* [127], *L. braziliensis* [128] and more currently from *L. donovani* [130] have been demonstrated with similar biological properties and location near the flagellar pocket region in promastigotes and megasomes of amastigotes [128, 133, 134].

L. chagasi, the causative agent of visceral leishmaniasis in Latin America also shows serine protease activities [135]. Three serine proteases named as LCSI, LCSII and LCSIII were isolated from extract of *L. chagasi* [129] exhibiting similar compartmentalization and substrate specificities with the serine proteases of other *Leishmania* species.

The role of serine proteases in visceral leishmaniasis caused by *L. donovani* is little known. An aprotinin sensitive *L. donovani* extracellular serine protease (pSP) of molecular mass 115 kDa was first identified [130] with their location in the flagellar pocket region in promastigotes and amastigotes (Fig. 3) [134]. Flow cytometry (Fig. 4) and confocal immunofluorescence (Fig. 3) analysis also revealed that the expression of the protease diminishes sequentially from virulent to attenuated strains of this species and is also highly associated with the metacyclic stage of *L. donovani* promastigotes [134]. Importantly pSP is upregulated during metacylogenesis and hopefully makes them important candidate as a participant in host-parasite interaction. Moreover, the pSP has strong proteolytic activity against extracellular matrix proteins, such as collagen and fibronectin [130], which suggests that the protease might be a superior agent for host tissue invasion and thus the role of pSP in host infection appears to be significant.

Besides the expression of secreted serine protease, a novel intracellular serine protease (SP-Ld) was also identified in *L. donovani* promastigotes [65]. This intracellular SP-Ld is also concentrated in the flagellar pocket region as well as on the surface

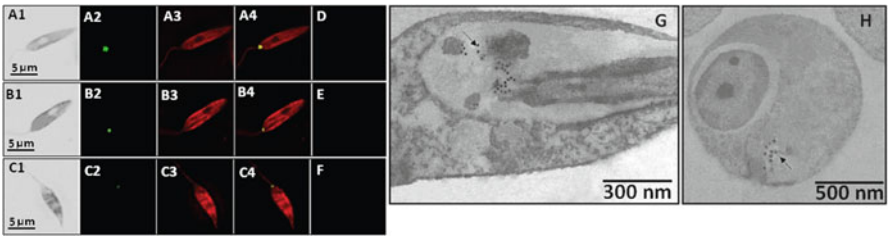

Fig. 3 The immunofluorescence images of Early-passage (*A1–A4*), late-passage (*B1–B4*), and UR6 (*C1–C4*) promastigotes of *L. donovani*. The promastigotes labeled for the pSP are shown in the *green* channel (*A2*, *B2*, and *C3*): GP63 are shown in the *red* (*A3*, *B3* and *C3*), Merged image in *yellow* channel (*A4*, *B4*, and *C4*). The phase-contrast image is shown on the *left* (*A1*, *B1* and *C1*). No Fluorescence was detected in similar preparations reacted with the preimmune serum (**d**, **e**, and **f**). Intracellular localization of the pSP of *L. donovani* by immunogold electron microscopy; the presence of gold particles in the flagellar pocket regions of the parasites Promastigotes (**g**) and amastigotes (**h**) indicated by *arrows* [134]

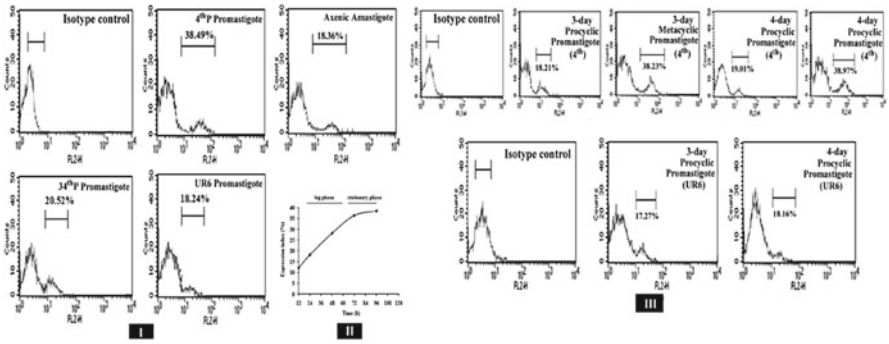

Fig. 4 Flow cytometric analysis of expression of the pSP. (*I*) Fluorescence histograms show the expression levels of the pSP in 4th-P, 34th-P, and UR6 promastigotes and axenic amastigotes of *L. donovani*. (*II*) Expression of the pSP of 4th-P promastigotes of *L. donovani* at different phases of growth. (*III*) Fluorescence histograms showing the expression levels of the pSP at procyclic and metacyclic stages of virulent promastigotes and at procyclic stages of attenuated UR6 promastigotes at 72 and 96 h of culture [134]

of the parasite (Fig. 5). The major role of SP-Ld could be predicted in invasion process as it down regulates the phagocytic activity of macrophages (Fig. 6) [65].

Using biochemical and molecular strategies two other serine proteases were also identified and characterized in *L. donovani* promastigotes which are of subtilisin [136] and oligopeptidase B [121] type.

During differentiation from promastigote to amastigote, OPB is upregulated in *Leishmania* and regulate levels of enolase on the parasite cell surface facilitating parasite entry into macrophages [121]. The direct effect of OPB on the host immune system was shown by examining the effect of infection with an OPB mutant strain on the expression of host genes. Infection of macrophages with a wild type

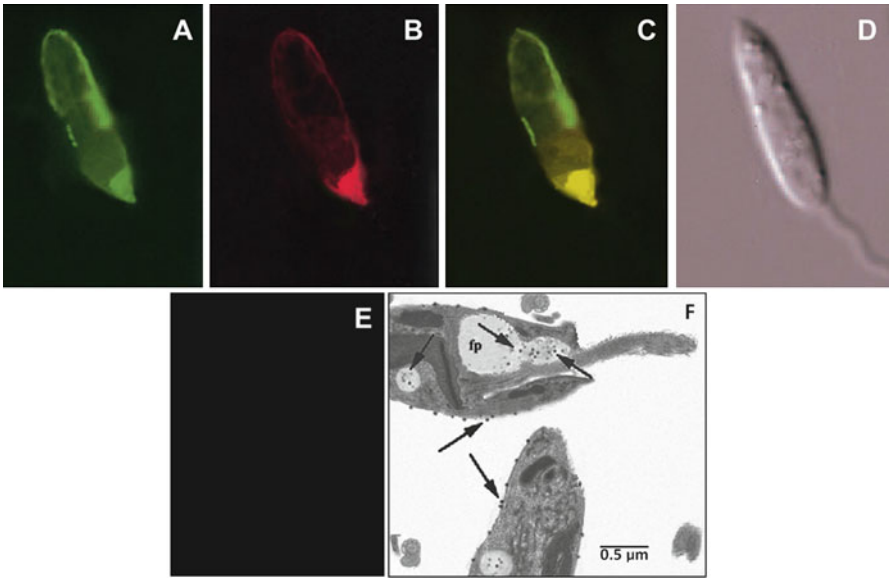

Fig. 5 Localization of *L. donovani* intracellular serine protease (SP-Ld) by confocal immunofluorescence and immune-gold electron microscopy. SP-Ld was envisioned by FITC-labeling (**a**), TRITC conjugated con A was used to label the flagellar pocket (*red*) (**b**), Merged images showing co-localization of SP-Ld within the flagellar pocket (**c**) and (**d**) is the phase contrast image of SP-Ld. No fluorescence was detected in presence of corresponding pre-immune serum (**e**). The presence of gold particles represents SP-Ld within flagellar pocket, cytoplasmic vesicles as well as at the surface of the *L. donovani* promastigotes indicated by the *arrows* (F) [65]

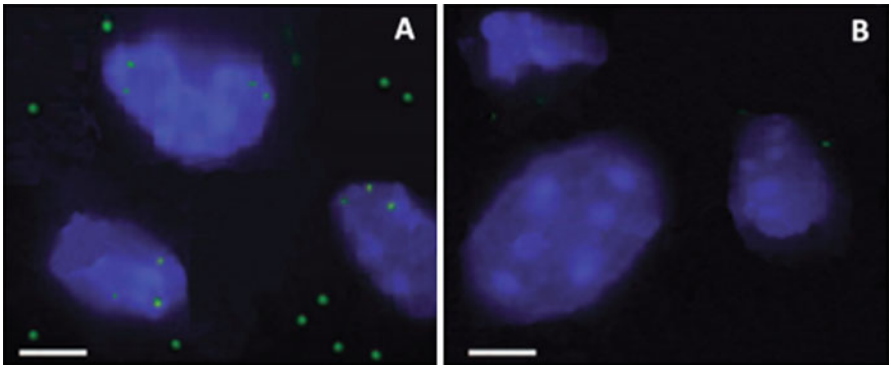

Fig. 6 Phagocytic activity of macrophages. FITC-coupled latex beads were co-incubated with macrophages in absence (**a**) and in presence of anti SP-Ld (**b**) treated parasites. Scale bars, 1 **µ**m [65]

L. donovani strain alters expressions of 23 genes, but infection with a mutant strain in which the oligopeptidase B gene was deleted leads to changes in 495 genes. This proves that OPB is necessary for *Leishmania* to silently infect macrophages [121]. Furthermore, these OPB (−/−) parasites displayed decreased virulence toward

mammalian host and suggested that *Leishmania* OPB itself is a prevalent virulence factor and also acts in conjunction with other factors [137].

The subtilisin protease (SUB; Clan SB, family S8) from *Leishmania donovani* was found to possess a unique catalytic triad and SUB-deficient *Leishmania* displayed reduced ability to undergo differentiation from promastigote to amastigote with some deformities i.e. abnormal membrane structures, retained flagella and increased binucleation [136]. On the basis of proteomic analysis, it has been reported that subtilisin is the maturase for tryparedoxin peroxidases to detoxify reactive oxygen intermediates for the maintenance of redox homeostasis and that is essential for *Leishmania* virulence [136]. Moreover, the activity of this serine protease is higher by several folds in amastigotes compared to promastigotes, suggesting an important role for this enzyme in parasites inside the host cells [136].

Serine proteases from *L. amazonensis* directly activated Th2 type immune response, and increased susceptibility to infection but, this effect was successfully eliminated in presence of specific serine protease inhibitors but not cysteine protease inhibitors [138]. So, *L. amazonensis* serine proteases exaggerate the infection by promoting Th2 type immune response. It was predicted that the *L. amazonensis* amastigote extract (LaE) containing serine protease is responsible for exacerbation of *Leishmania* infection by promoting Th2-type immune responses [139].

Besides being important targets of drug development against *Leishmania*, serine proteases are also vaccine candidates for leishmaniasis. Recently, Choudhury et al. [140] have shown that the *L. donovani* extracellular serine protease (pSP) confer significant protection in experimental visceral leishmaniasis (VL) and in this study the vaccine efficacy of pSP was further investigated for its prophylactic potentiality by regulating host MMP-9 profile. Hence, it can be postulated that *Leishmania* proteases may participate in modification of macrophages functions by modulating matrix metalloproteinase activity. Currently, available data suggest that serine proteases and MMPs might play essential functions in development of leishmaniasis and help researchers to investigate the miscellaneous roles of these proteases to design effective therapeutic strategies against leishmaniasis.

2.4 Aspartic Proteases

The presence of aspartic protease was first reported in *Leishmania* in 2005 [129]. It was present at its highest level in promastigotes and in the early stages of differentiation to amastigotes [135]. *L. major* genome contains two aspartic peptidases [66]. One has similar sequence with presenilin 1 (PS1), a multi-pass membrane peptidase, and is able to cleave type I membrane proteins [34]. Another one has identical sequence with an intramembrane signal peptide peptidase (SPP) which cleaves the transmembrane domains of signal peptidases [66]. PS1 has been implicated to be involved in autophagy in *L. major* [66]. An aspartic protease activity was also identified and characterized in *L. mexicana* promastigotes [141]. A recent study has reported that Ddi1-like protein is functional aspartyl proteinase in *L. major* and it

can be a possible potential target for novel antiparasitic drugs [142]. The antiproliferative effect of its inhibition makes this enzyme a putative new target for the development of leishmanicidal drugs. In this context, Savoia and co-workers in 2005 [143] first demonstrated the impressive effects of indinavir and saquinavir on the growth of *L. major* and *L. infantum*. Later, it was demonstrated that HIV aspartyl-protease inhibitors (HIV-PIs) powerfully reduce *L. infantum* infection in macrophages, either co-infected or not with HIV [144]. In addition, this activity was target of antiproliferative effect on *Leishmania* promastigotes and axenic amastigotes by HIV-PIs, Ac-Leu-Val-Phenylalaninal, saquinavir mesylate and nelfinavir [145]. A direct action of these HIV-PIs on *Leishmania* parasites opens an interesting standpoint for new drugs research development based on this novel parasite protease for the treatment of HIV/Leishmania co-infection [146]. In addition, HIV-PIs also hampered *L. amazonensis* growth and their interaction with macrophages, indicating that the HIV-PIs are active against a wide range of *Leishmania* species and probably induce several serious ultrastructural modifications in *L. amazonensis* promastigotes. This effect of HIV-PIs is terminated with parasite death, may be due to a disproportion between apoptosis and autophagy [147]. This dose-dependent inhibition of *Leishmania* aspartyl-protease activity by these drugs certainly validates the possible association between aspartic protease expression and basic molecular processes in *Leishmania*. Despite all these beneficial effects, the HIV-PIs induced an increase in the expression of CPB and GP63 [148]. So, further investigations are essential to control HIV/*Leishmania* co-infection. However, the noticeably increasing numbers of *Leishmania* and HIV co-infected patients and direct effect of the HIV-PIs on opportunistic pathogens support researchers to seek for direct effects of HIV-PIs on *Leishmania* [40, 149, 150].

3 Proteases as Drug Targets in Leishmaniasis

Leishmaniasis remains a challenge for public health due to lack of effective vaccine and thus as of now, chemotherapy is the only alternative for controlling the disease [26, 27]. The current treatments available are greatly disappointing due to high cost, toxicity and widespread resistance and therefore the present situation needs worldwide development of potential new drugs to combat leishmaniasis. One of the main features in the drug development is to identify a possible target of biological pathway of parasite life cycle, in a view of that the target should be either absent in the host or be unique from the host homologous proteins so that it can be exploited as a putative drug target. It has also been described that one of the characteristic features in the process of drug development is to identify the putative target [151]. In this context, proteases would be excellent objects because of their vital roles in parasitic biology [40, 152]. Hence, investigations are currently underway to elucidate their possible function by means of protease inhibitors. The protease inhibitors inactivate or block proteolytic enzymes by binding to its active site or by other mechanisms

[153] hindering one or several fundamental events caused by the enzymes and thus, uses of protease inhibitors also enhance our knowledge about the biological function of the enzymes in the parasite physiology. Therefore, the main approach has been to achieve good inhibitors of the target protease, in the faith that inhibition of the protease activity of the pathogen will be of therapeutic value.

Parenthetically proteases have being authenticated as druggable targets in many cases [27, 154–156], and protease inhibitors are also being broadly investigated to develop therapeutic drugs against cancer, cardiovascular, inflammatory, neurodegenerative, bacterial, viral and parasitic diseases due to important roles played by the proteases in these diseases [157–160]. Hence, the ongoing progress in the design of protease inhibitors may also present a challenge for advanced therapies of parasitic diseases [155, 161–164]. Protease inhibitors thus have potential utility for therapeutic interposition in a variety of disease states including trypanosomasis and leishmaniasis [159]. However, as the parasites are eukaryotes, treatments of trypanosomatid diseases are difficult by means of protease inhibitors as antiparasitics because they may lead to the host toxicity and possible adverse side effects. But, current research on drug design makes it feasible for formulation of specific protease inhibitors with negligible cross reactivity and of great potentiality [40, 159].

For instance, the proteasome of *Leishmania* is a potential therapeutic target as inhibition of proteasome blocks parasite growth [165]. Besides, several research groups have suggested that cysteine proteases in *Leishmania* may also be very promising target [36, 71, 166, 167]. *Leishmania* treated with CP inhibitors showed reduced viability, growth and pathogenicity [70, 71]. In addition, treatment with a natural CP inhibitor, cystatin, promoted a protective response against *Leishmania* infection and a switch from a predominately Th2 to a Th1-type of immune response [72, 92, 93]. The CP inhibitors have also been shown to prevent the activation of a latent form of TGF-β, a known suppressive cytokine in *Leishmania* infections [168]. Moreover, it has been found that both metallo and cysteine peptidase inhibitors could hinder the growth of *L. braziliensis* as well as the association index with macrophages [169]. Thus, presently, a lot of researches are progressing to develop potent cysteine protease inhibitor as an antileishmanial drug. Unlike cysteine protease, a little research has been focused on *Leishmania* GP63 to evaluate it as a drug target. Previously, it has been demonstrated that development of higher affinity metalloproteases inhibitors may provide a novel approach for treatment of parasitic diseases [170]. *Leishmanias* lacking GP63 are unable to activate the host PTPs for sabotage host cell signaling and lose their ability to sustain infection [59]. Therefore, it can be clearly speculated that potent and specific inhibitors of GP63 could be able to trigger the host antimicrobial functions and thus would gain the accessibility of future antileishmanial therapeutics [40]. Nevertheless, due to immunogenicity and antigenicity of *Leishmania* GP63, many studies have been performed to evaluate its efficacy as vaccine against *Leishmania* [84, 171].

Currently, the possibility of implication of HIV protease inhibitors against *Leishmania* has raised interest due to increasing rate of HIV and *Leishmania* co-infection in certain regions of the world and protease inhibitors are being extensively used to treat HIV and *Leishmania* co-infected patients [172–175]. Recently,

some compounds are now being experimentally used to treat leishmaniasis targeting particular protease [176, 177].

Serine protease inhibitors have also been used by many investigators in parasitic diseases in searching potent drug [117, 178, 179] as serine protease activity can be regulated in the cells or in the organisms by employing specific protease inhibitors [180, 181]. These inhibitors are valuable tools for investigation of the biochemical properties and the biological functions of the proteases [182, 183]. In addition, invasion blockage of many parasites, including *Plasmodium falciparum* [184, 185], *Babesia divergens* [117], *Toxoplasma gondii* [186] and *Perkinsus marinus* [187] have been observed by using specific serine protease inhibitors. Previous reports have shown that pentamidine and suramin, exhibit trypanocidal activity through the inhibition of the cytosolic serine protease oligopepetidase B, a putative virulence factor in trypanosome [188, 189].

Initially, serine protease inhibitors were used to evaluate the possible functions of serine proteases in *Leishmania* [190]. The effect of serine peptidase inhibitors on the survival of *Leishmania* has shown that TPCK (N-tosyl-l-lysyl-chloromethylketone) and benzamidine both reduces viability and induce morphological changes in the *Leishmania amazonensis* promastigotes, raising the possibility that serine peptidases could be useful potential drug targets [190]. Moreover, treatment with different type of serine protease inhibitors, especially with aprotinin, which block the active site of the protease, resulted in marked reduction in cellular viability [190]. Specific doses of these compounds stimulate significant morphological modifications in the flagellar pocket region accompanied by forming bleb that coats the flagellar pocket [190]. These effects indicate that serine protease inhibitors are probably introduced through this structure and inhibited the serine proteases in this pocket region. Moreover, these serine proteases inhibitors induce the formation of autophagic vacuoles and destroy *Leishmania* promastigotes. Although serine proteases are essential for parasite survival, their function in *Leishmania* physiology remains to be illuminated. However, this is the first evidence where *Leishmania* serine proteases have been emerged to be another promising target for the development of antileishmanial chemotherapy. Moreover, the treatment of *L. amazonensis* promastigotes antigens (LaAg) with irreversible serine protease inhibitors reversed its disease-promoting effect [138] which is again another indication of exploitation of serine proteases in the development of antileishmanial drugs [152, 190, 191].

Although, several *Leishmania* serine proteases have been characterized, their function in the parasite physiology is still under investigation. Based on studies from our laboratory and other groups, serine proteases seem to play essential roles in infection process and deactivating the macrophage during the initial interaction between the host and the parasite [65, 121, 134, 136–140]. Altogether, these characteristics of *Leishmania* serine proteases strongly suggest that potent and specific inhibitors of serine protease could instigate the activation of host's antimicrobial properties and thereby leading to destruction of parasites. Hence, the possible approach of using specific serine protease inhibitors as prophylactic drugs could be able to inhibit the onset of *Leishmania* infection [40].

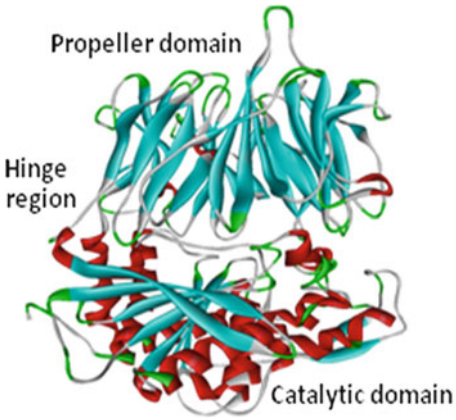

Fig. 7 Crystal structure of OPB [PDB ID 2XE4] done by McLuskey et al. [111]: The tertiary structure of OPB with antipain bound in the active site. General loop regions are shown in *yellow*. The hinge regions are between the two domains and the catalytic domain is represented by deep colors

But, uses of serine protease inhibitors in treatment of leishmaniasis need extensive studies to understand the roles of serine proteases in parasite physiology and pathogenesis. In some reports, it has been postulated that structure based drug design could be achieved by means of three dimensional model e.g. in *L. amazonensis* oligopeptidase B (Fig. 7) [111]. It has also been proposed that prolyl oligopeptidase and oligopeptidase B, both members of the S9 serine protease family would be admirable choice for drug design against Chagas disease, leishmaniasis and African trypanosomiasis [192, 193]. In addition to the computational design, development and optimization of a suitable protease inhibitor, based on the 3D structure of the target protease will be valuable tools for investigation of the biochemical properties and functions of proteases as well as in the treatment of leishmaniasis. On the other hand, ongoing researches on *Leishmania* proteases are still expanding our knowledge on parasite biology, particularly with a great concern over current concept of serine proteases and demonstrating them to be potential drug targets. Therefore, as a whole, the developments of both synthetic and natural protease inhibitors have relevant importance in the search of new therapeutic alternatives for leishmaniasis. Eventually, the development of protease inhibitors of particular parasitic proteases will be the best option for improved understanding of physiological significance of the proteases in disease pathogenesis as well as to identify them as good candidates for antileishmanial therapy.

4 Conclusion

Leishmania gets advantages from various virulent factors especially from proteases. leishmanial proteases help invasion and survival in intra or extracellular environments of the host. So, proteases are considered as potential drug targets in *Leishmania*

parasite For instances, *Leishmania* GP63 and cysteine protease subvert host immune response throughout various mechanisms. Aspartic protease is an important virulent factor in case of *Leishmania*-HIV co-infected patients. In this context, roles of *Leishmania* serine proteases need to be further defined because some recent reports suggest that this protease also perform crucial roles in parasite physiology and in the host-parasite interaction. For instance, Oligopeptidase B (OPB) is increasingly being implicated as an important virulence factor in leishmaniasis. Elucidation of the substrate specificity and regulation of OPB activity paved the way to develop drugs that are specific for the parasite. It was also observed that specific serine protease inhibitors alters parasite morphology and reduced the viability, growth of *Leishmania* and also causes death of both extracellular and intracellular parasite. Hence, protease inhibitors must be considered to be promising candidates for drug development in the leishmaniasis treatment and it would be a rational approach toward other parasitic diseases as well.

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