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A possible molecular mechanism for parasitic inhibition by Lactoferrin

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A POSSIBILE MOLECULAR MECHANISM FOR PARASITIC INHIBITION BY LACTOFERRIN

Summary

Transferrins (Tfs) belong to a family of iron-binding glycoproteins possessing similar aminoacid sequence though they have different biological functions and locations. Lactoferrin (Lf) is expressed and secreted from glandular epithelial cells and from mature neutrophiles of mammalian and it is an important component of the aspecific host defence or natural immunity, including resistance to parasitic infections. Serum transferrin (sTf) is synthesized by the liver of mammals and secreted into the blood stream; its primary function is iron transport. Ovotransferrin (Otrf), synthesized by avians, displays both iron transport and protective functions.

Parasites synthesize papain-like cysteine proteases that are relevant for the virulence and pathogenicity of parasites, being involved in several aspects of the parasite life cycle, it is therefore possible that the antiparasitic activity of Lf could be due to the inhibition of parasitic papain-like cysteine protease that we have recently observed.

In this study we have investigated the thermodynamic parameters of hLf, bLf and Otrf inhibition of the parasitic papain-like type I cysteine proteases from *Leishmania infantum*, *Trypanosoma cruzi* and *Trypanosoma brucei*. bLf, hLf and Otrf, both in the apo- and olo-forms, showed time- and concentration-dependent inhibition of the catalytic activity of papain and of type I proteases from *L.infantum*, *T. cruzi* and *T. brucei*. The K_I values observed for bLf and hLf inhibition of *L. infantum*, *T. cruzi* and *T. brucei* proteases were in the nanomolar range (K_I = 3.1 nM), lower than K_I values observed for papain inhibition (K_I = 24 nM). Otrf showed lower inhibition of cysteine proteases (K_I = 0.6 μM). On the contrary, sTf did not display any inhibition towards parasitic proteases, according to its different role in mammals. The inhibition of parasitic cysteine proteases by hLf, bLf and Otrf appeared to conform to a competitive mechanism. The observed pH

optimum for bLf inhibition of parasitic proteases was around neutrality, while it was acidic for hLf and alkaline for Otrf. The further quantitative analysis of pH dependence of the intrinsic ligand-independent inhibition constant K_I allowed the evaluation of pK_a values that define the acid-base equilibrium of amino acidic residue(s) modulating the enzyme(s)-inhibitor recognition events. SDS-PAGE showed that hLf, bLf and Otrf were easily degraded by either papain or parasitic type I protease during the assay incubation time (few minutes) and it is likely that one or more protease inhibitory peptides were generated from protein hydrolysis.

As a matter of fact, a sequence present near the C-terminus of human (hLf) and bovine (bLf) lactoferrin shows homology with the sequence of the active site of cystatins, which are competitive inhibitors of papain-like cysteine proteases. The same sequence is present, though with lower homology, in Otrf and, with even lower homology, in sTf.

Therefore, we have charactherized by MALDI-TOF the profile of Lf cleavage by papain and preliminary data suggest the presence of a cystatin-like peptide in two proteolytic fragments of hLf and in one proteolytic fragment of bLf.

Le transferrine (Tfs) sono una famiglia di glicoproteine in grado di legare reversibilmente il ferro, e presentano un'elevata omologia di sequenza tra tutti i membri di questa famiglia. In particolare la lattoferrina (Lf) è prodotta e secreta nelle cellule ghiandolari epiteliali ed è presente nei granuli dei granulociti neutrofili dei mammiferi. Essa rappresenta un'importante componente della difesa immunitaria aspecifica dell'ospite. La siero transferrina (sTf) invece è sintetizzata nel fegato, ed è coinvolta unicamente nel trasporto del ferro. L'ovotransferrina (Otrf), l'omologa aviaria della Lf , svolge sia una funzione di difesa che di trasporto del ferro.

Le proteasi a cisteina papaina-simili da protozoi e metazoi parassiti, secretorie e di membrana, partecipano ai processi di morfogenesi degli organismi patogeni, sono implicate nell'invasione delle cellule e dei tessuti da parte dei parassiti, riducono la risposta immunitaria dell'ospite e costituiscono uno dei fattori più rilevanti nelle patologie associate alle infestazioni da parassiti. Pertanto, a fronte della capacità della Lf di svolgere un'azione antiparassitaria, noi abbiamo ipotizzato che la Lf possa svolgere tale azione grazie all'inattivazione delle suddette proteasi a cisteina.

Nel presente lavoro abbiamo studiato le proprietà termodinamiche dell'inibizione delle proteasi a cisteina di alcuni parassiti, quali *Leishmania infantum*, *Tripanosoma cruzi* e *Trypanosoma brucei.* Tale studio è stato esteso anche verso la papaina, capostipite di questa famiglia. Innanzitutto è emerso una maggiore affinità delle transferrine studiate (bLf, hLf e Otrf, in forma apo e olo) verso le proteasi parassitarie (K_I = 3.1 nM) rispetto a quella verso la papaina (K_I = 24 nM). Otrf risulta essere l'inibitore con minore affinità (K_I = 0.6 μM). Inoltre è emerso che la sTf non è in grado di svolgere una'zione inibitoria, coerentemente con la sua funzione nei mammiferi. L'inibizione di tutte le proteasi risulta essere conforme ad un meccanismo di inibizione competitivo.

Da un'analisi della dipendenza del pH dei suddetti fenomeni di inibizione si è osservato una maggiore attività della bLf a pH fisiologico mentre la hLf inibisce più efficacemente a valori di pH acidi (pH = 5.0) e la Otrf a valori di pH alcalini (pH = 9.0). La dipendenza dal pH del parametro K_I relativo all'interazione degli inibitori studiati con gli enzimi, ha permeso di evidenziare la variazione di diversi valori di pK_a, ascrivibili ad eventi di protonazione e deprotonazione di differenti gruppi ionizzabili.

Inoltre si è osservato, a seguito di SDS-page, una parziale idrolisi della Lf e della Otrf durante diverse incubazioni con la papaina e con le proteasi parassitarie, e l'insieme di tali dati suggerisce la liberazione di un peptide, che può quindi inibire competitivamente le suddette proteasi a cisteina.

A supporto di tale ipotesi, si è osservato in presenza della porzione C-terminale della Lf, una sequenza aminoacidica che presenta un'elevata omologia con il sito attivo delle cistatine, una (super)famiglia di proteina, riconosciute essere gli inibitori reversibili per eccelenza delle proteasi a cisteina papaina-simili. Abbiamo quindi caratterizzato, mediante MALDI TOF, il profilo di idrolisi della Lf ad opera della papaina, ed è stata riscontrata la presenza di frammenti contenenti tale sequenza cistatino-simile, sia nella bLf che nell'hLf.

Abbreviations

ACN	Acetonitrile
apo-Lf,	lactoferrin in its iron free state (Fe^{3+})
bLf,	bovine lactoferrin
CEW Cys	chicken egg-white cystatin
CHCA	α-Cyano-4-hydroxycinnamic acid
CMV,	*Cytomegalovirus*
DTT,	(-)-**1**,**4**-Dithiothreitol
DMSO,	dimethylsulfoxide
HBV,	*Human hepatitis B*
HCV,	*Human hepatitis C*
hLf,	human Lactoferrin
holo-Lf,	lactoferrin iron bound (Fe^{3+})
HSV,	*Herpes simplex virus*
Lf,	lactoferrin
Otrf,	ovotransferrin
sTf,	human serum transferrin
Tf,	transferrin
TFA	Trifluoroacetic acid
Z-Phe-Arg-AMC,	*N*-α-benzyloxycarbonyl-L-phenylalanyl-L-arginine-(7-amino-4-methyl coumarin)

Introduction

Among parasitic Protozoa, trypanosomatids belonging to the *Leishmania* and *Trypanosoma* genres comprise etiological agents of endemic diseases mainly localised in developing countries. All the human pathogenic trypanosomatids are dixenic parasites: namely with a complex life-cycle characterized by a vector host (an ematophagous insect) which became infected after a blood meal on a infected mammalian and which is responsible to bear and to transmit the flagellate parasite to the next mammalian host. The last one becoming in turn a source of infection for further insect vectors (Cox, 1993; Gilles, 1999; WHO, 2002).

In particular, *Trypanosoma cruzi* is the agent of the American trypanosomiasis (Chagas' disease), affecting at least 20 million people, being responsible of the chagasic cardiopathy, the most relevant clinical manifestation of Chagas' chronic disease, affecting about a third of the infected people (Cox, 1993; Gilles, 1999; WHO, 2002).

Tripanosoma brucei is the causative agent of African sleeping sickness (human African trypanosomosis), a vector-borne disease that is fatal if untreated (Barrett *et al.*, 2003) and ranks second among parasitic diseases in sub-Saharan Africa only to malaria in terms of mortality (Cox, 1993; Gilles, 1999).

Furthermore, several species of the genre *Leishmania* (i.e. those belonging to the *L. mexicana*, *L. brasiliensis*, *L. dononvani* and *L. tropica* complexes) cause a broad spectrum of diseases, affecting 12 million of people in both the Old and New Worlds. Leishmaniasis can be fatal (visceral leishmaniasis), grossly disfiguring (mucocutaneous leishmaniasis), or relatively mild, localized, and in some cases self-healing (some forms of cutaneous leishmaniasis) (Cox, 1993; Gilles, 1999; WHO, 2002).

These obligate parasites have two hosts - an insect vector and mammalian host. Because of the large difference between these hosts the trypanosome undergoes complex changes during its life cycle to facilitate its survival in the insect gut and the mammalian bloodstream (Barret *et al*., 2003; Matthews, 2005).

In the last years growing evidences arose pointing to the importance between the parasitic cisteine proteases and the virulence and pathogenicity of parasites. In fact, cysteine proteases belonging to the

papain family are relevant to several aspects of the parasite life cycle and of the parasite-host relationship and are thus seen as promising therapeutic target of parasitic diseases. As an example, the major papain-like cysteine proteases from *T. Cruzi*, *T. brucei* and *L. infantum*, are mainly expressed in all stages of the parasites life cycle. Among others, cruzipain participates in the penetration of *T. cruzi* trypomastigotes into host cells, in the nutrition of the parasite at the expense of the host, and in the escape mechanisms of the parasite from the immune system of the host. Similar biological roles, even though with distinct molecular mechanisms, are been proposed for cysteine proteases from *Leishmania* (Coombs & Mottram, 1997; Del Nery *et al*., 1997; McKerrow, 1999; McKerrow *et al*., 1993, 1995, 1999; Frame *et al*., 2000; Caffrey *et al*., 2000; Lecaille *et al*., 2002; Sajid & McKerrow, 2002; Mottram *et al*., 2004). In fact the cysteine proteases of *L. mexicana* are critical in suppressing protective immune responses, inhibiting host TH1 responses (Buxbaum *et al*., 2003).

1.1. Cysteine proteases: the papain family

Cysteine proteases are divided into four main groups referred to as clans, CA, CB, CC and CD. Conventionally, proteases are assigned to clans and families depending on a number of characteristics including sequence similarity, common peptide loops, and substrate specificity to small peptide substrates. The clans CB and CC include viral proteases, while in the clan CD (Legumain-like family) there are asparagynil peptidase and transamidase. Asparaginyl endopeptidases exclusively hydrolyse peptides and proteins on the carboxyl side of asparagines residues. These enzymes are often referred to as ‘legumain-like’ as the template protease was first identified and characterised from the plant legume, *Canavalia ensiformis*, the jack bean. Legumain-like proteases have been identified in many plants, mammals including, human, mouse, rat and pig, and in many parasite organisms like *Fasciola hepatica*, *Schistosoma mansoni* and *Caenorhabditis elegans* (Lecaille *et al*., 2002; Sajid & McKerrow, 2002; Mottram *et al*., 2004).

1.1.1 Papain-like proteases

In 1879 the first cysteine protease was purified and characterised from *Carica papaya*, the papaya fruit, and was thus named papain. Papain was also the first cysteine protease structure to be solved. Since its discovery, numerous proteases that have sequences in common with papain have been loosely called 'papain-like'(Lecaille *et al.*, 2002).

Recently, on the base of structural and catalytic properties, the papain-like proteases have been divided in three different subfamilies (Lecaille *et al.*, 2002; Sajid & McKerrow, 2002; Barrett *et al.*, 2004).

Members of the Cathepsin L-like subfamily have a conserved inter-spaced motif in the pro-region, Glu-X_3-Arg-X_2-Phe-X_2-Asn-X_3-Ile-X_3-Asn ('ERFNIN'; named after the single letter code for amino acids; X is any amino acid) and dispaly an endopeptidase activity (Lecaille *et al.*, 2002; Sajid & McKerrow, 2002; Barrett *et al.*, 2004).

Members of the subfamily Cathepsin B-like lack the ERFNIN motif but do have an inserted peptide loop in the catalytic domain, referred to as the 'occluding loop', between residues Tyr103 and Cys128 (human cathepsin B-like numbering). In addition to endopeptidase activity (like cathepsin L), cathepsin Bs have a dipeptidyl carboxypeptidase activity. This has been attributed to the occluding loop (Lecaille *et al.*, 2002; Sajid & McKerrow, 2002; Barrett *et al.*, 2004).

Members of the Cathepsin F-like family, the latest enzymes included in papain-like family, have a consensus motif in the pro-region Glu-X_3-Arg-X_2-Phe-X_2-Asn-X_3-Ala-X_3-Gln/Ala ('ERFNAQ'; named after the single letter code for amino acids) (Wex *et al.*, 1999, 2000; Barrett *et al.*, 2004).

1.1.2. Structural aspects and catalytic mechanism

The enzyme is assembled from two domains, each comprising residues from both the N- and C-terminal sections of the polypeptide. One domain consists of a six-stranded antiparallel β-sheet (R-domain) and contains the catalytic residues histidine (His159: papain numbering) and asparagine (Asn175: papain numbering) (Fig. 1) (McGrath, 1999; Sajid & McKerrow, 2002; Barrett *et al.*, 2004).

The L-domain, instead, consists mainly of three α-helices. The cysteine residue (Cys25 based on papain numbering) is embedded in a highly conserved peptide sequence, Cys-Gly-Ser-**Cys**-Trp-Ala-Phe-Ser (active site cysteine residue in bold) (Fig. 1) (Rawlings & Barrett, 1994; Turk *et al*., 1997, 1998; Barrett *et al*., 2004).

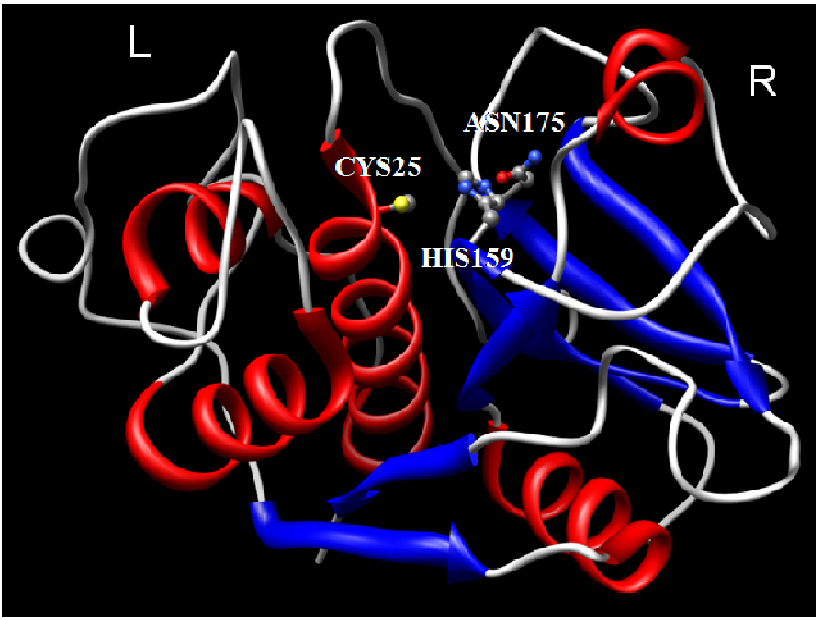

Figure 1: Structure of papain (code PDB: 9PAP) (Kamplius *et al.*, 1994). Molecular graphics images were produced using the UCSF Chimera package.

The catalytic site of papain-like cysteine proteases is highly conserved and formed by three residues: Cys25, His159, and Asn175 (papain numbering). Cys25 and His159 form an ion pair which is stabilized by Asn175 *via* a hydrogen bond. This triad has some similarities to the active site present in serine proteases (Ser, His, Asp). However, in contrast

to serine proteases the nucleophilic cysteine residue is already ionized prior to substrate binding and thus cysteine proteases can be regarded as *a priori* activated enzymes (Polgard & Halasz, 1982).

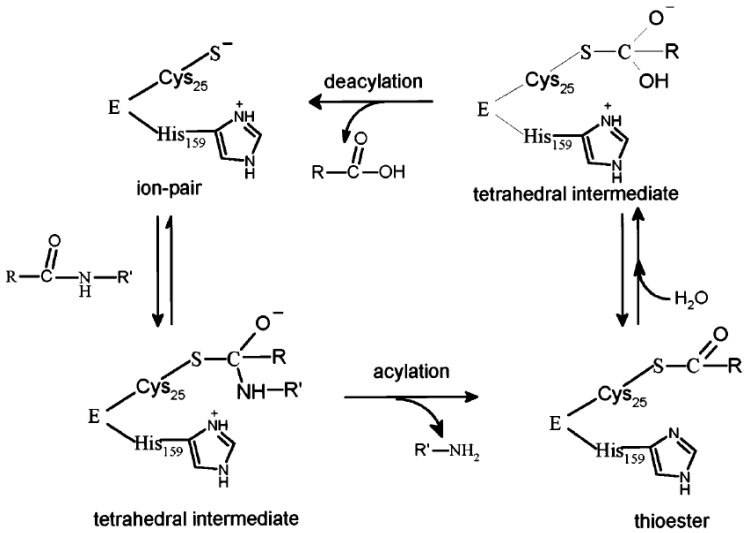

Figure 2: Mechanism of substrate hydrolysis by papain-like cysteine proteases (Lecaille *et al*., 2002).

During peptide hydrolysis, the nucleophilic thiolate cysteine attacks the carbonyl carbon of the scissile bond of the bound substrate and forms a tetrahedral intermediate which is stabilized by the so-called oxyanion hole (Ménard *et al*., 1991). The tetrahedral intermediate transforms into an acyl enzyme (enzyme-substrate thiol ester) with the simultaneous release of the C-terminal portion of the substrate (acylation). This step is followed by the hydrolysis of the acyl enzyme with water, forming a second tetrahedral intermediate which finally splits into the free enzyme and the N-terminal portion of the substrate (deacylation) (Storer & Ménard, 1994, Vernet *et al*., 1995) (see Fig. 2).

1.1.3. Cruzipain and Brucipain

Trypanosoma cruzi is a protozoan parasite which is transmitted to humans, as an infectious trypomastigote form, from the bite of a blood-sucking insect. The trypomastigote enters the host bloodstream and ultimately invades a cardiac muscle cell, where it transforms into the intracellular amastigote. Amastigotes replicate within cells, transform back to trypomastigotes, and rupture the cell, releasing the infectious form back into the bloodstream and other cells, amplifying the infection (Duschak & Couto, 2009).

Cysteine proteases appear to be relevant to several aspects of the *T. cruzi* life cycle and the parasite-host relationship. In particular, cruzipain (Fig. 3), the major cysteine protease from *T. cruzi* epimastigotes, is expressed in all stages of the parasite life cycle, being more abundant in replicating forms and particularly in the insect epimastigote stage. Notably, cruzipain participates in the penetration of *T. cruzi* trypomastigotes into host cells, in the nutrition of the parasite at the expense of the host, and in the escape mechanisms of the parasite from the immune system of the host (Duschak & Couto, 2009).

Cruzipain has a structure very similar to that of papain. In fact is composed of polypeptide chain of 215 amino acid residues, folded into two domains (Figure 3). One domain is mainly α-helical (L-domain, residues 12-112 and 208-212), and the other consists of extensive antiparallel β-sheet interactions (R domain, residues 1-11 and 113-207) (Kamphuis *et al*., 1984, 1985; McGrath *et al*., 1995). The catalytic residues Cys25, His159 and Asn175, and the extended substrate-binding site, are found in a cleft between the two domains (McGrath *et al*., 1995).

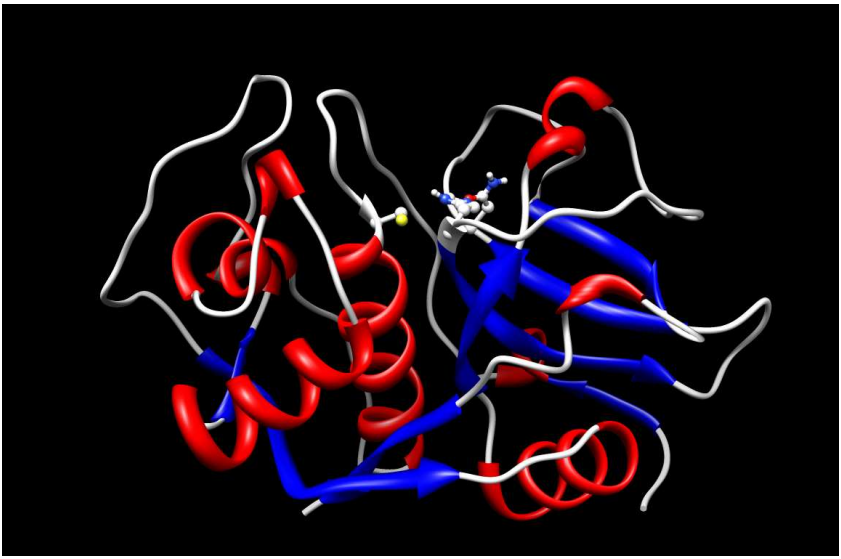

Figure 3: Structure of Cruzipain (code PDB: 1AIM) (Gillmor *et al.*, 1997). Molecular graphics images were produced using the UCSF Chimera package.

Trypanosoma brucei is a parasitic protist species that causes African trypanosomiasis (or sleeping sickness) in humans and in animals in Africa. There are 3 sub-species of *T. brucei*: *T. b. brucei*, *T. b. gambiense* and *T. b. Rhodesiense* (Barret *et al*., 2003).

In *T. brucei* species, the major cysteine protease has primary sequence and biochemical characteristics that are broadly similar to those of mammalian cathepsin L-like (Lonsdale-Eccles & Grab, 1987; Troeberg *et al*., 1999; Caffrey *et al*., 2001), and is encoded by a tandem array of 11 nearly identical gene copies (Berriman *et al*., 2005). The enzymes in *T. b. rhodesiense* and *T. b. brucei* are termed rhodesain and brucipain (or trypanopain) respectively (Lonsdale-Eccles & Grab, 1987; Caffrey *et al*., 2001 Mackey *et al.*, 2004).

Brucipain shows 80% homology and 60% identity with the sequences of cruzipain (Duschak & Couto, 2009).

1.1.4. Protease of type I from *L. infantum*

The proteases of type I from *L. infantum* is a glycoprotein, synthesized like pre-pro-enzyme and it has a N-domain of 27 amino acidic residues (aa), a pre-peptidic element of 98 aa, a catalytic domain of 214 aa and a C-domain of 104 aa (Souza *et al*., 1992; Mottram *et al*., 1997; Campos-Ponce *et al*., 2005).

Like cruzipain, the proteases from *L.infantum* are cathepsins L-like and these proteases are required for parasite replication and virulence (Sakanari *et al*., 1997; Campos-Ponce *et al*., 2005; Mundodi *et al*., 2005).

Sequence analysis showed that they are 90% identical to *L. major* Cathepsin B, 66% identical to *L. mexicana* cathepsin B, and 45% identical and 57% homology to cruzipain, indicating high sequence conservation among the trypanosomatid species (Mottram *et al*., 1997).

1.2. Cystatins family

The inactivation of parasite cysteine proteinase mediated by synthetic inhibitors block replication and differentiation of *T. cruzi*, *T. Brucei* and *L. infantum* both *in vitro* and *in vivo*, providing an alternative to traditional therapy in drug-resistant parasites (Coombs & Mottram, 1997; Maekawa *et al.*, 1998; McKerrow, 1999; McKerrow *et al.*, 1993, 1995, 1999; McKerrow, 1999; Caffrey *et al.*, 2000; Frame *et al*., 2000; Lecaille *et al*., 2002; Lima *et al*., 2002; Sajid & McKerrow, 2002; Somanna *et al.*, 2002; Ascenzi *et al.*, 2004).

Cysteine protease inhibitors (CPI) belonging to the cystatin (super)family inactivate proteases by trapping them in a(n) (ir)reversible, tight equimolar complex (Barrett *et al*., 1998). The representatives of this group of CPI is characterized by a wide distribution, being present in

mammals, birds, fish, insects, plants and some protozoa (Abrahamson *et al.*, 2003).

The human superfamily of cystatins is divided into three families. Family I, called stefins, comprises intracellular cystatins A and B. Family II includes extracellular and/or transcellular cystatins (cystatins: C, D, E, F, S, SA, and SN). Kininogens, the intravascular cystatins, form family III of cystatins (Barrett, 1987; Rawlings & Barrett, 1990; Turk & Bode, 1991).

All cystatins have the same structure consisting of a five stranded β-sheet wrapped around a five turn α-helix. Cystatins contain three segments which are recognized as responsible for the interaction with cysteine proteases. These are the N-terminal fragment and the so-called first and second loops (L1 ans L2, respectively), which are arranged at one edge of the molecule and are believed to directly interact with the catalytic cleft of cysteine proteases (Grzonka *et al*., 2001; Turk *et al.*, 2008).

These three cystatin regions, containing evolutionarily conserved amino-acid residues, form a wedge-like structure, which interacts with the catalytic cleft of cysteine proteases. These interactions are hydrophobic binding between the regions of cystatins and the corresponding residues forming the binding pockets of the enzyme. In particular, I-loop contains the conserved residues QVVAG, that interact with the active site of cysteine proteases, whereas the N-terminal fragment contains the conserved residues Leu9 and Gly11 (human cystatin numbering) (Grzonka *et al*., 2001; Turk *et al.*, 2008). These residues form the reactive syte of cystatins.

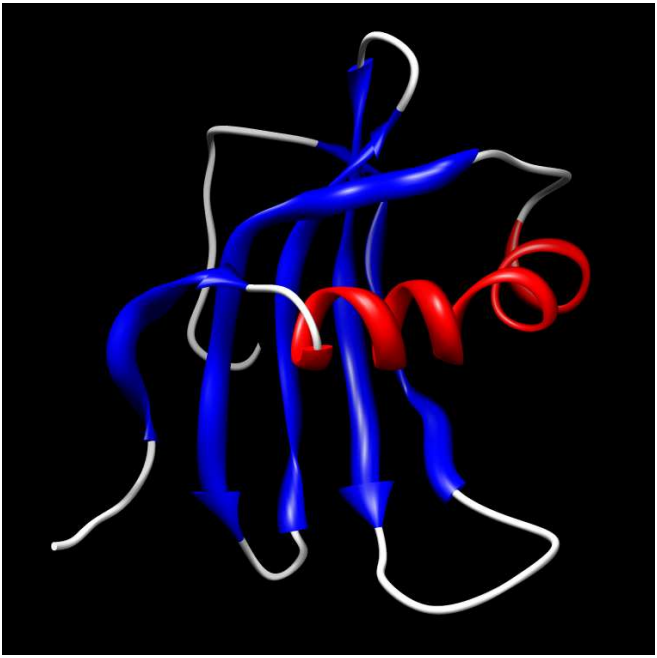

Figure 4: Structure of Stefin A (code PDB: 1DVD) (Gillmor *et al.*, 1997). Molecular graphics images were produced using the UCSF Chimera package.

Recently a sequence present at the C-terminus of human (hLf) and bovine (bLf) lactoferrin, a mammalian glycoprotein, shows a homology with the sequence of the reactive site of the cystatins (Katunuma *et al.*, 2003). Moreover, this sequence is present in Ovotrasnferrin (Otrf), avian homologous of Lf, and in serum Transferrin (sTf), synthesized in the liver of mammals and secreted into the blood (Anderson *et al.*, 2009).

Table 1: Alignment of Lf sequence with cystatine active site

Cystatin active site		**LG QVVAG**	
bLf	(680)	**LGTEYVTA**	(687)
hLf	(683)	**LGPQYVAG**	(690)
Otrf	(657)	**LGDKFYTV**	(664)
sTf	(661)	**LGEEYVKA**	(688)

1.3. Lactoferrin

Lactoferrin (Lf) is a non-haem iron-binding protein that is part of the transferrin protein family, along with serum transferrin (sTf), ovotransferrin (Otrf), melanotransferrin and the inhibitor of carbonic anhydrase, whose function is to transport iron in the blood serum (González-Chávez *et al*., 2009).

LF is produced by mucosal epithelial cells in various mammalian species, including humans, cows, goats, horses, dogs and several rodents. Recent studies have shown that Lf is also produced by fish, as it has been identified in rainbowtrout eggs using molecular biology techniques (González-Chávez *et al*., 2009).

This glycoprotein is found in mucosal secretions, including tears, saliva, vaginal fluids, semen (van der Strate *et al*., 2001), nasal and bronchial secretions, bile, gastrointestinal fluids, urine (Öztas & Özgünes, 2005) and most highly in milk and colostrum (7 g/L) (Rodriguez *et al*., 2005) making it the second most abundant protein in milk (Connely, 2001), after caseins. It can also be found in bodily fluids such as blood plasma and amniotic fluid. Lf is also found in considerable amounts in secondary neutrophil granules (15 µg/10^6 neutrophils) (Bennett & Kokocinski, 1987; González-Chávez *et al*., 2009), where it plays a significant physiological role.

Lf possesses a great iron-binding affinity and is the only transferring with the ability to retain the metal over a wide pH range (Aisen & Leibman, 1972) including extremely acidic pH. It also exhibits a great

resistance to proteolysis. In addition to these differences, Lf net positive charge and its distribution in various tissues make it a multifunctional protein (Baker & Baker, 2009).

1.3.1 Structure

Lf (Fig. 5) is an80 kDa glycosylated protein of ca. 700 aminoacids (711 aa for hLf and 689 aa bLf) with high homology among species. It is a simple polypeptide chain folded into two symmetrical lobes (N and C lobes) which are highly homologous with one another (33–41% homology) (Anderson *et al.*, 1987, 1989; Baker, 1994; Moore *et al.*, 1997; Sharma *et al.*, 1998; Baker & Baker, 2009).

Figure 5: Structure of hLf (code PDB: 1FCK) (Baker *et al.*, 2000). Molecular graphics images were produced using the UCSF Chimera package.

These two lobes are connected by a hinge region containing parts of an α-helix between residues 333 and 343 in human Lf (hLF), which provides additional flexibility to the molecule. The polypeptide chain includes amino acids 1–332 for the N lobe and 344–703 for the C lobe and is made up of α-helix and β-pleated sheet structures that create two domains for each lobe (domains I and II) (Moore *et al.*, 1997). Each lobe can be further divided into two subdomains (N1 and N2 in the N-lobe and C1 and C2 in the C-lobe) that form a cleft inside of which the iron is bound. The subdomain N1 contains residues 1-90 and 251-333, while N2 contains the residues 91-250) (Baker *et al.*, 1987; Moore *et al.*, 1997; Baker & Baker, 2009).

Each lobe can bind a metal atom in synergy with the carbonate ion (CO_3^{2-}). Lf binds notably Fe^{2+} and Fe^{3+} ions, but also Cu^{2+}, Zn^{2+} and Mn^{2+} ions (Aisen & Harris, 1989; Baker *et al.*, 1994, 2005; Baker & Baker, 2009).

1.3.2 Functions of Lf

Lf is involved in several physiological functions, including: regulation of iron absorption in the bowel; immune response; antioxidant, anticarcinogenic and anti-inflammatory properties. Protection against microbial infection, whichis the most widely studied function to date (Sanchez *et al.*, 1992; Brock, 1995; Lonnerdal & Iver, 1995; Vorland, 1999; Brock, 2002; Antonini *et al.*, 2005; Baker & Baker, 2009; Leboffe *et al.*, 2009). The antimicrobial activity of LF is mostly due to two mechanisms. The first is iron sequestration in sites of infection, which deprives the microorganism of this nutrient, thus creating a bacteriostatic effect. The other mechanism is the direct interaction of LF with the infectious agent. Positively charged amino acids of LF can interact with anionic molecules on some bacterial, viral, fungal and parasite surfaces, causing cell lysis (Bullen, 1981; Braun & Braun, 2002; Valenti & Antonini, 2005).

Considering the physiological capabilities of Lf in host defence, in addition to current pharmaceutical and nutritional needs, Lf is considered to be a nutraceutical and for several decades investigators have searched for the most convenient way to produce it (González-Chávez *et al.*, 2009).

The antiviral activity of hLf was first demonstrated in mice infected with the polycythemia-inducing strain of the Friend virus complex (FVC-P) (Lu *et al.*, 1987). Since 1994, potent antiviral activity of hLf and bLf has been demonstrated against both enveloped and naked viruses, like *Cytomegalovirus* (CMV) (Harmsen *et al*., 1995; Andersen *et al*., 2001), *Herpes simplex virus* (HSV) (Marchetti *et al*., 1996, 1998; Siciliano *et al*., 1999; Valenti & Antonini, 2005), *Human immunodeficiency virus* (HIV) (Swart *et al*., 1996 ; Puddu *et al*., 1998; Berkhout *et al.*, 2004), as well as *Human hepatitis C* (HCV) and *human hepatitis B* (HBV) viruses (Ikeda *et al*., 1998; Hara *et al.*, 2002).

Molecular mechanims of Lf antiparasitic activity are even more complex. Antiparasitic activities of Lf appear often to involve interference with iron acquisition by some parasites, e.g. *Pneumocystis carinii*, while Lf appears to act as a specific iron donor in other parasites such as *Trichomonas foetus;* in the latter case, Lf could be expected to enhance infection. It was recently reported that two *T. brucei* proteins bind human serum transferrin as well as human and bovine Lf. Preincubation of *Toxoplasma gondii* and *Eimeria stiedai* sporozoites with a Lf-derived peptide, lactoferricin, reduces their infectivity in animal models. Lf antiparasitic activity is also, sometimes, mediated by interaction with host cells. Thus, iron-saturated Lf enhances intramacrophage killing of *T. cruzi* amastigotes and decreases intra-erythrocytic growth of *Plasmodium falciparum*. Lf is able to inhibit the invasion of cultured cells by *Plasmodium* spp. sporozoites through specific binding to HS. In the case of *Plasmodium berghei*, Lf reduces invasion by inhibiting the binding of the plasmodial CS protein, with or without HS, suggesting the possibility that Lf can also bind to the same site on LDL receptor-related protein (LRP) as the CS protein (see Leboffe *et al.*, 2009).

AIM

The protozoan order Kinetoplastida includes the genus *Trypanosoma*, members cause some of the most neglected human diseases, the trypanosomiases. There are many species of trypanosome, and the group infects most vertebrate genera. Several trypanosoma species cause important veterinary diseases, but only two cause significant human diseases (Gilles, 1999). In sub-Saharian Africa, *Trypanosoma brucei* causes sleeping sickness or human African trypanosomiasis, and in America, *Trypanosoma cruzi* causes Chagas' disease (Gilles, 1999). The kinetoplastida also contains species of the genus Leishmania that cause a range of diseases in the tropics and subtropics (Gilles, 1999). All microrganisms are transmitted by insect vectors. The transmission in the human host follows blood transfusion, contamined needles, biting insects, vector faeces, and contamined food (Gilles, 1999).

Like all microorganisms, the genus *Trypanosoma* has an absolute necessity of iron to sustain their growth (Loo & Lalond, 1984; Leboffe *et al*,, 2009). In particular, some parasites (e.g. *Trypanosoma cruzi*) survive in mammalian blood and could utilize serum mammalian chelates directly. Other parasites, such as *leishmania* spp., survie intracellularly in mammals (Gilles, 1999). Therefore they have envolved a mechanism to utilize mammalian holo-transferrin (Tf) and/or holo-Lactoferrin (Lf) via several means.

The Tf uptake by *T. brucei* has been well characterized. In this protozoan, esag6 and esag7 genes encoded for a protein associated to a heterodimeric transferrin-binding protein complex (TFBP) present on the cell membrane (Leboffe *et al.*, 2009). These receptors have the ability to bind Tf from different mammalian host species (Leboffe *et al.*, 2009). Fluorescence and immunoelectron microscopy showed that the *T. brucei* TFBP–Tf complex is internalized and transported to lysosomes, where Tf is proteolytically degraded to release iron (Leboffe *et al.*, 2009).

On the contrary, the promastigote uptake of iron for *Leishmania* is different and involves reduction of extracellular iron. Infact holo-Tf binds to a binding protein on the promastigote surface, as well as for *Trypanosoma brucei*. One electron is donated to Fe^{3+} through the action of a membrane-associated or secreted iron reductase complex. The complex could include a cytoplasmic NADPH-requiring reductase which has specificity for NADPH

and which donates an electron to a membrane electron carrier (Leboffe *et al.*, 2009). Fe^{2+} has a very low affinity for Tf and therefore dissociates easily (Baker, 2009).

In the last years growing evidences arose pointing to the importance between parasitic cisteine proteases and the virulence and pathogenicity of trypanosomatid parasites. In fact, cysteine proteases belonging to the papain family are relevant to several aspects of the parasite life cycle and of the parasite-host relationship and are thus seen as promising therapeutic target of parasitic diseases (Coombs & Mottram, 1997; Del Nery *et al*., 1997; McKerrow, 1999; McKerrow *et al*., 1993, 1995, 1999; Frame *et al*., 2000; Caffrey *et al*., 2000; Lecaille *et al*., 2002; Sajid & McKerrow, 2002; Mottram *et al*., 2004).

As an example, the major papain-like cysteine proteases from *T. cruzi* and *L. infantum* are mainly expressed in all stages of the parasites life cycle. Among others, cruzipain participates in the penetration of *T. cruzi* trypomastigotes into host cells, in the nutrition of the parasite at the expense of the host, and in the escape mechanisms of the parasite from the immune system of the host. Similar biological roles, even though with distinct molecular mechanisms, are been proposed for cysteine proteases from *Leishmania* (Coombs & Mottram, 1997; Del Nery *et al*., 1997; McKerrow, 1999; McKerrow *et al*., 1993, 1995, 1999; Frame *et al*., 2000; Caffrey *et al*., 2000; Lecaille *et al*., 2002; Sajid & McKerrow, 2002).

Notably, the inactivation of parasite cysteine proteinases by synthetic inhibitors block replication and differentiation of *T. cruzi* and *L. infantum* both *in vitro* and *in vivo*, providing an alternative to traditional therapy in drug-resistant parasites (Coombs & Mottram, 1997; Maekawa *et al*., 1998; McKerrow, 1999; McKerrow *et al*., 1993, 1995, 1999; McKerrow, 1999; Caffrey *et al*., 2000; Frame *et al*., 2000; Lecaille *et al*., 2002; Lima *et al*., 2002; Sajid & McKerrow, 2002; Somanna *et al*., 2002).

Cysteine protease inhibitors (CPI) belonging to the cystatin (super)family inactivate proteases by trapping them in a(n) (ir)reversible, tight equimolar complex (Barrett *et al*., 1998). The representatives of this group of CPI is characterized by a wide distribution, being present in mammals, birds, fish, insects, plants and some protozoa (Abrahamson *et al*., 2003; Turk *et al.*, 2008).

Recently it was demonstrated that human and bovine milk has a new function against cysteine proteases (Katunuma *et al*., 2003). These data strongly support the hypothesis that Lf antiparasitic activity could be due, at least partially, to the inhibition of parasitic proteases.

Therefore, in the present work we report a quantitative investigation on the thermodynamic parameters for Tf inhibition of the cysteine protease from *L. infantum*, cruzipain and brucipain by hLf and bLf. Moreover, Lf binding to papain has been investigated. Moreover, the influence of the pH on the inhibition constants of Lf with papain and with the protease from *L. Infantum* have been determinated. The analysis of data allowed the identification of putative aminoacidic residues that may presumably modulate the interaction of the cysteine proteases studied with the Lf. Since this sequence is present also in Otrf (ovotransferrin), avian homologous of Lf, we have investigated the Otrf binding to protozoan proteases.

Results and discussion

3.1. Thermodynamic characterization of papain and cysteine proteases from *L. Infatum*, *T. cruzi* and *T. brucei*

3.1.1 The catalytic mechanism

As shown in Fig. 6, the hydrolysis of the fluorogenic substrate Z-Phe-Arg-AMC catalyzed by the papain follows the simple Michaelis-Menten mechanism depicted in Scheme I and defined by equation (1).

Altought similar to the papain, the cysteine proteases from *T. cruzi*, *T. brucei* and *L. infantum* display the phenomenon of the substrate inhibition. In fact, as can be seen from the dependence of the relative initial velocity (i.e.: v_i/V_{max}) of Z-Phe-Arg-AMC hydrolysis on the substrate concentration, at 25 °C and pH 6.0 (Fig. 6) under steady state conditions (i.e.: [E] < 5 × [S]), the parasite enzymes follow the substrate inhibition mechanism described by Scheme II and defined by equation (2) (Fig.6: theoretical curves as solid lines and experimental data as squares and rumbles for the cysteine proteases from *T. Cruzi*, *T. Brucei* and *L. infantum*, respectively).

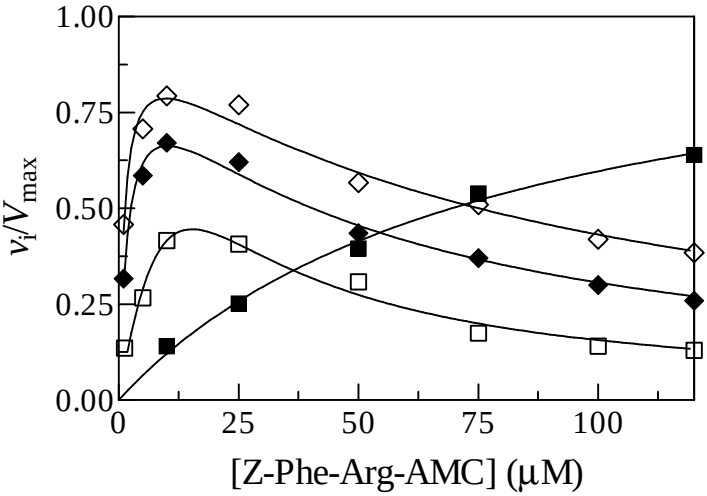

Figure 6: Effect of the substrate concentration on the hydrolysis of Z-Phe-Arg-AMC catalyzed by papain (black squares), cruzipain (black rumbles), the protease from *L. infantum* (rumbles) and brucipain (squares). Data were obtained at pH 6.0 and 25 °C. Solid lines, representing the theoretical curves, were calculated through an iterative non-linear least squares procedure with sets of parameters given in Table 2 and Table 3, according to Equation (1) for papain and to Equation (2) for cysteine proteases from *T. Cruzi*, from *T. Brucei* and from *L. infantum*. The standard deviation for each experimental point was equal to ± 8%. For further details, see text.

Table 2: Values of K_m (μM) for the hydrolysis of Z-Phe-Arg-AMC by proteases from *L. infantum, T. cruzi, T . brucei* and by papain (25,0°C).

pH	Papain	Cruzipain	Brucipain	Prot. from *L. infantum*
4.0	225±21	4.4±0.3	42.9±0.8	10.9±0.8
5.0	94±8	3.0±0.2	31.4±0.5	5.7±0.8
6.0	70±7	1.6±0.1	16.0±0.3	2.7±0.8
7.0	80±9	1.3±0.1	8.8±0.2	1.4±0.8
8.0	116±10	1.1±0.1	8.4±0.2	1.1±0.8
9.0	224±15	1.1±0.1	8.2±0.1	1.1±0.8

Table 3: Values of K_i (μM) for the hydrolysis of Z-Phe-Arg-AMC by the proteases from *T. cruzi*, *T. brucei* and *L. infantum* and (25.0°C).

pH	Cruzipain	Brucipain	Protease from *L. infantum*
4.0	-----*	------*	33±0.8
5.0	48±8	55±0.2	36±0.5
6.0	59±7	68±0.1	40±0.3
7.0	67±9	77±0.1	55±0.2
8.0	73±10	84±0.1	62±0.2
9.0	74±15	85±0.1	57±0.1

* At such pH value cruzipain and brucipain do not undergo to substrate inhibition phenomenon.

Although the molecular details of substrate inhibition of cysteine proteases are not known, it often occur with synthetic and small substrates (Cazzulo *et al*., 1990; Lima *et al*., 1992; Stoka *et al*., 1998; Venturini *et al*., 2000; Salvati *et al*., 2001a). These chemicals are supposed to be able to bind the cysteine proteases interacting with only few residues of the protein and thus leading to the possibility for the synthetic molecule to assume different

configurations in respect with the catalytic Cys25 residue (mature cruzipain numbering). On the geometry of the interaction will then depend the result of the substrate-enzyme reaction (Cleland, 1979). Therefore, Z-Phe-Arg-AMC presumably is capable to bind the active site of parasite enzymes with, at least, two alterative configurations: either productively (S_s, Scheme II), leading to the hydrolysis of the substrate itself, or following a binding mode that lead to the enzyme inhibition (S_i, Scheme II) (Salvati *et al*., 2001a; Salvati *et al*., 2002). *Vice versa*, papain, although with an active site very similar in the structure to the ones held by the parasite proteases, can interact with Z-Phe-Arg-AMC only through a productive configuration (S_s, Scheme I) (Szabelski *et al*., 2001) These considerations agree with the different specificity that characterizes papain, on one side, and parasite cysteine proteases from *T. cruzi*, *T. brucei* and from *L. infantum*, on the other. It has been proposed that the occurrence of the substrate inhibition phenomenon can reflect the presence of Glu residue (Glu205, mature cruzipain numbering) lying at the bottom of the S2 sub-site of the active site of papain-like cysteine proteases (as in the case of cruzipain, brucipain and of the protease from *L .infantum*) while the corresponding papain residue (Ser) should not be able to permit to the synthetic molecule to assume alternative (and inhibitory) geometries (see Salvati *et al*., 2001a). Remarkably, Glu205 was found to be capable of adopting different conformations according to the nature of the pairing P2 residue, thus enabling the parasite cysteine proteinase to bind substrates and inhibitors with charged (i.e. Arg) or hydrophobic (i.e. Tyr and Phe) residues at their P2 position (Gillmor *et al*., 1997; McGrath *et al*., 1995; Polticelli *et al*., 2005). Accordingly, if Z-Phe-Arg-AMC binds productively to the cruzipain active centre (i.e. Phe(P2) and Arg(P1) residues fit the S2 and S1 clefts, respectively), then it acts as substrates. On the other hand, if the Arg(P1) residue or the Z and AMC substituents of Z-Phe-Arg-AMC fit the S2 recognition sub-site of cruzipain, the substrate inhibition occurs (i.e. Z-Phe-Arg-AMC acts as an inhibitors) (Salvati *et al*., 2001a).

3.1.2 Effect of pH on values of K_m and K_i

The analysis of the experimental data according to equation (1) and (2) for papain and parasite cysteine proteases action, respectively, allowed the evaluation, independently from the V_{max} estimation, of the Michaelis constant (K_m, for all the four proteases, Fig. 7 and Table 2) and of the substrate inhibition constant K_i (related only to the parasite enzymes, Fig. 8

and Table 3) for the hydrolysis of the Z-Phe-Arg-AMC between pH 4.0 to 9.0.

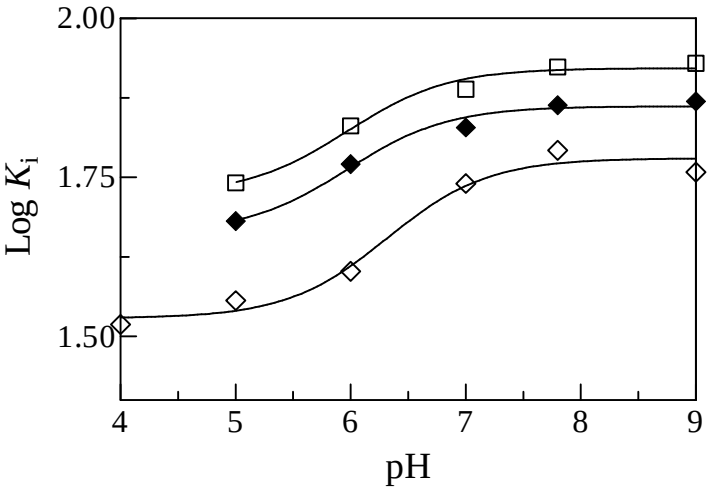

Figure 7: Effect of pH on values of K_m for the hydrolysis of Z-Phe-Arg-AMC catalyzed by papain (black squares), cruzipain (black rumbles), the protease from *L. infantum* (rumbles) and brucipain (squares). Solid lines represent the best fit of experimental data was calculated, according to Equation (3) for parasite cysteine proteases and according to Equation (4) for papain, through an iterative non-linear least squares procedure with sets of parameters given in Table 1. The standard deviation for each experimental point is given in Table 1. The analysis allowed the determination of the p*K*' and p*K*'' parameters given in Table 3. For further details, see text.

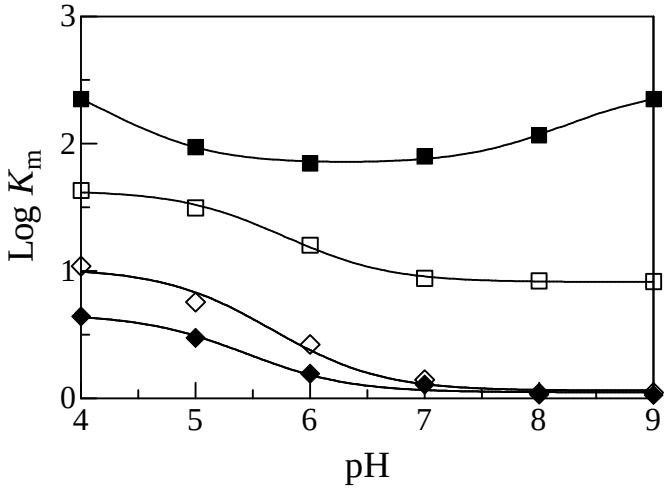

Figure 8: pH dependence of the substrate inhibition constant K_i (μM) for the hydrolysis of the Z-Phe-Arg-AMC catalyzed by cruzipain (black rumbles), *L. infantum* protease (rumbles) and brucipain (squares) at 25,0 °C. Solid lines, representing the best fit of experimental data calculated according to Equation (5) through an iterative nonlinear least squares procedure with sets of parameters given in Table 2. Standard deviation for each of the experimental point is reported in Table 2. For pH values below pH 5 cruzipain does not show the substrate inhibition. The analysis allowed the determination of the p*K*° parameters given in Table 3. Standard deviation regarding experimental points is given in Table 2. For further details, see text.

Table 4: Values of pK_a parameters which describe the pH dependence of the Michaelis constant K_m (in black) and the pH dependence of the substrate inhibition constant K_i (in blue) for the hydrolysis of the Z-Phe-Arg-AMC by papain and by the cysteine proteases from *L. Infantum*, from *T. cruzi* and from *T. brucei* (25,0°C).

	pK_a Values	
Papain	pK'$_{unl}$ = 4.6±0,1	pK'$_{lig}$ = 3.9±0,2
	pK"$_{unl}$ = 7.9±0,1	pK"$_{lig}$ = 8.5±0,1
Cruzipain	pK'$_{unl}$ = 5.7±0,1	pK'$_{lig}$ = 5.1±0,1
	pK°$_{unl}$ = 5.9±0,2	pK°$_{lig}$ = 6.1±0,2
Brucipain	pK'$_{unl}$ = 6.1±0,1	pK'$_{lig}$ = 5.4±0,1
	pK°$_{unl}$ = 5.9±0,2	pK°$_{lig}$ = 6.1±0,2
Protease	pK'$_{unl}$ = 6.1±0,2	pK'$_{lig}$ = 5.2±0,2
from *L. infantum*	pK°$_{unl}$ = 6.2±0,2	pK°$_{lig}$ = 6.4±0,2

Values of K_m for the papain (Fig. 2) depend on the acid-base equilibrium of two ionising residues. The analysis of data allowed to determine the values of pK'$_{unl}$ (= 4.6±0.1), pK'$_{lig}$ (= 3.9±0.2), pK"$_{unl}$ (= 7.9±0.1) e pK"$_{lig}$ (= 8.5±0.1) (Table 5) and values of pK_a reflecte the equilibrium of two amino acidic residue (Fig. 7 and Table 4), classically recognised as the catalytic residues Cys25 (*i.e.* pK'$_{unl}$ = 4.6±0.1 and pK'$_{lig}$ =

3.9±0.2) and His159 (i.e. pK''$_{unl}$ = 7.9±0.1 and pK''$_{lig}$ = 8.5±0.1) (Hollaway *et al*., 1969; Shipton *et al*., 1976; Ascenzi *et al*., 1987; Ménard *et al*., 1990). Nonetheless, the influence of further residues lying in the active site of the enzyme, like Lys156 and Asp158 can not be excluded and they may account for minor contributions in the enzyme-substrate binding energetic, at least modifying the electric field acting on catalytic residues Cys25 and His159 and thus on their observed pK_a values.

On the other hand, the analysis of data concerning cruzipain, brucipain and *L. infantum* protease action (Fig. 7) allowed the estimation of the pK_a values that modulates the protease-substrate binding event, for cruzipain (pK'$_{unl}$ = 5.7±0.1 e pK'$_{lig}$ = 5.1±0.1), brucipain (pK'$_{unl}$ = 6.1±0.1 e pK'$_{lig}$ = 5.4±0.1) and for the *L. infantum* protease (pK'$_{unl}$ = 6.1±0.1 e pK'$_{lig}$ = 5.2±0.2) (Table 4).

Therefore, while Z-Phe-Arg-AMC binding to papain imply a shift of the apparent pK_a of 0.7 unit toward acidic pH values (pK'$_{unl}$ > pK'$_{lig}$) and of 0.6 unit toward the alkaline (pK"$_{unl}$ < pK"$_{lig}$) the interaction of Z-Phe-Arg-AMC with cruzipain, brucipain and *L. infantum* protease determines a shift of the apparent pK_a to acidic values (pK'$_{unl}$ > pK'$_{lig}$) of about 0.7 pH units.

On the other hand, only one apparent amino acidic residue is able to modulate the interaction of Z-Phe-Arg-AMC with cruzipain and with *L. Infantum* protease regarding to the productive as well as to the unproductive binding (S_S and S_i respectively, see Scheme II). Notably, for all the parasite enzymes the ligand-independent pK_a values which define the pH dependence of both the productive (pK'$_{unl}$) and the non-productive binding process (pK°$_{unl}$) converge in the limits of the experimental indeterminateness (i.e. for cruzipain and for *L. Infantum* protease it results pK'$_{unl}$ ~ pK°$_{unl}$, see Table 3). These values for cruzipain (pK'$_{unl}$ = 6.2±0.2, pK°$_{unl}$ = 6.2±0.2) are very close to those that characterize *L. Infantum* protease (pK'$_{unl}$ = 5.7±0.1, pK°$_{unl}$ = 5.9±0.2). The main structural determinant that may account for the observed pH dependence of Z-Phe-Arg-AMC binding to parasite cysteine proteases is probably the Glu205 residue (mature cruzipain numbering). As reported above, this residue, Glu in parasite proteases and Ser in papain, is responsible for the different specificity showed by papain and by parasite cysteine proteases. Notably, previous investigations on cysteine proteases from *T. cruzi*, *L. infantum* and *Plasmodium falciparum* (Ascenzi *et al*., 2004). Human cathepsin B (Khouri *et al*., 1991) and viral cathepsin from the *Autographa californica* nuclear

polyhedrosis virus (AcNPV) (Bromme & Okamoto, 1995) have shown similar results with reference to the pH dependence of the binding of synthetic ligands to papain-like proteases with a Glu residue equivalent to the Glu205 of cruzipain. It seems to suggest a general model of recognition and of pH-modulation for cysteine proteases with a cathepsin B-like S2 specificity sub-site (Sajid & McKerrow, 2002).

3.2. Inhibition of papain and related proteases by members of the Transferrin family

3.2.1. Tfs are a competitive inhibitors

Lf and Otrf (Tfs) inhibit papain, cruzipain, brucipain and *L. infantum* protease (Fig. 9 and Table 5). The inhibition of cysteine proteases by Lf and Ortf conforms to competitive inhibition according to the classic competitive inhibition mechanism (Scheme III) described by Equations (6), (7) and (8). On the contrary, sTf does not inactive cysteine proteases, according to its different role in mammals (Anderson *et al.*, 2009).

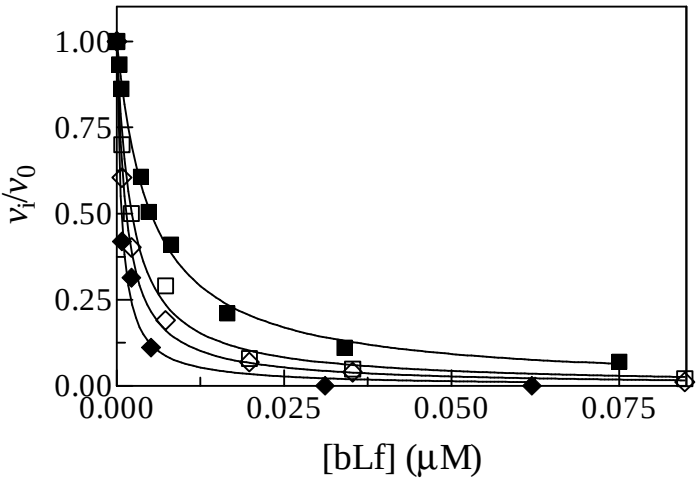

Figure 9: Dependence of the initial relative velocity v_i/v_0 for the hydrolysis of Z-Phe-Arg-AMC by papain (black squares), cruzipain (black rumbles), *L. infantum* protease (rumbles) and brucipain (squares) on the bLf concentration. Experimental data are been obtained at pH 6.0 and 25 °C. The Z-Phe-Arg-AMC concentration was 50 μM for papain and 5.0 for parasite cysteine proteases. Solid lines represent the theoretical curves, described by Equation (6), obtained through an iterative non-linear least squares procedure with sets of parameters given in Table 5. Standard deviation for each experimental point was equal to ± 8%.

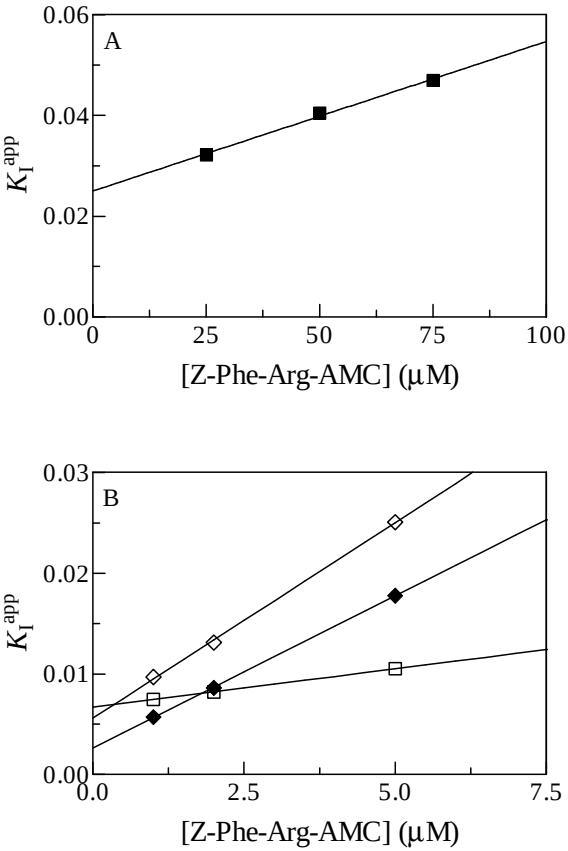

Figure 10: Dependence of the relative inhibition constant K_{Iapp}/K_I by bLf binding to papain (Panel A, black squares), with cruzipain (Panel B, black rumbles), *L. infantum* protease (Panel b, rumbles) and brucipain (Panel B, squares) on the substrate concentration (Z-Phe-Arg-AMC). Experimental data were carried out at 25°C and pH 6.0. Straight lines, representing the best fit of experimental data, were calculated according to Equation (8) through an iterative linear least squares procedure with sets of parameters given in Tables 5-8. Standard deviation for each experimental point was to ± 8%.

Table 5: Values of K_I (μM) for bLf, hLf (holo and apo) and Otrf binding to papain (25.0 °C).

pH	bLf	hLf-holo	hLf-apo	Otrf
4.0	$(3.5\pm0.1)\times10^{-1}$	$(7.1\pm0.6)\times10^{-2}$	$(8.5\pm0.8)\times10^{-2}$	$(9.8\pm0.1)\times10^{-1}$
5.0	$(2.2\pm0.1)\times10^{-1}$	$(7.2\pm0.6)\times10^{-2}$	$(9.1\pm0.8)\times10^{-2}$	$(8.6\pm0.1)\times10^{-1}$
6.0	$(5.1\pm0.1)\times10^{-2}$	$(7.6\pm0.6)\times10^{-2}$	$(9.3\pm0.1)\times10^{-2}$	$(7.0\pm0.1)\times10^{-1}$
7.4	$(2.4\pm0.1)\times10^{-2}$	$(1.6\pm0.1)\times10^{-1}$	$(2.0\pm0.1)\times10^{-1}$	$(4.6\pm0.1)\times10^{-1}$
8.0	$(4.7\pm0.3)\times10^{-2}$	$(3.4\pm0.2)\times10^{-1}$	$(4.3\pm0.1)\times10^{-1}$	$(3.5\pm0.1)\times10^{-1}$
9.0	$(7.4\pm0.6)\times10^{-2}$	$(5.1\pm0.4)\times10^{-1}$	$(6.3\pm0.1)\times10^{-1}$	$(1.2\pm0.1)\times10^{-1}$

Table 6: Values of K_I (μM) for bLf, hLf (holo and apo) and Otrf binding to *L. infantum* protease (25.0 °C).

pH	bLf	hLf-holo	hLf-apo	Otrf
4.0	$(2.5\pm0.1)\times10^{-2}$	$(2.0\pm0.1)\times10^{-2}$	$(3.5\pm0.2)\times10^{-2}$	$(9.9\pm0.8)\times10^{-1}$
5.0	$(2.0\pm0.1)\times10^{-2}$	$(1.8\pm0.1)\times10^{-2}$	$(3.3\pm0.2)\times10^{-2}$	$(8.4\pm0.1)\times10^{-1}$
6.0	$(1.0\pm0.1)\times10^{-2}$	$(1.2\pm0.1)\times10^{-2}$	$(2.2\pm0.1)\times10^{-2}$	$(6.9\pm0.5)\times10^{-1}$
7.4	$(6.0\pm0.5)\times10^{-3}$	$(9.0\pm0.8)\times10^{-3}$	$(1.7\pm0.1)\times10^{-2}$	$(4.5\pm0.3)\times10^{-1}$
8.0	$(8.8\pm0.7)\times10^{-3}$	$(1.2\pm0.1)\times10^{-2}$	$(2.3\pm0.1)\times10^{-2}$	$(2.6\pm0.1)\times10^{-1}$
9.0	$(1.6\pm0.1)\times10^{-2}$	$(2.3\pm0.1)\times10^{-2}$	$(4.3\pm0.3)\times10^{-2}$	$(1.3\pm0.1)\times10^{-1}$

Table 7: Values of K_I (μM) for bLf, hLf (holo and apo) and Otrf binding to cruzipain (25.0 °C).

pH	bLf	hLf-holo	hLf-apo	Otrf
4.0	$(9.7\pm0.8)\times10^{-2}$	$(3.2\pm0.1)\times10^{-2}$	$(2.0\pm0.1)\times10^{-2}$	$(9.8\pm0.1)\times10^{-1}$
5.0	$(3.3\pm0.1)\times10^{-2}$	$(2.6\pm0.1)\times10^{-2}$	$(1.6\pm0.1)\times10^{-2}$	$(8.9\pm0.1)\times10^{-1}$
6.0	$(6.8\pm0.6)\times10^{-3}$	$(1.3\pm0.1)\times10^{-2}$	$(8.2\pm0.7)\times10^{-3}$	$(5.0\pm0.1)\times10^{-1}$
7.4	$(3.1\pm0.1)\times10^{-3}$	$(7.5\pm0.6)\times10^{-3}$	$(4.7\pm0.1)\times10^{-3}$	$(1.6\pm0.1)\times10^{-1}$
8.0	$(5.8\pm0.4)\times10^{-2}$	$(1.1\pm0.1)\times10^{-2}$	$(7.0\pm0.6)\times10^{-3}$	$(6.3\pm0.5)\times10^{-2}$
9.0	$(2.5\pm0.1)\times10^{-2}$	$(2.0\pm0.1)\times10^{-2}$	$(1.3\pm0.1)\times10^{-2}$	$(4.0\pm0.3)\times10^{-2}$

Table 8: Values of K_I (μM) for bLf, hLf (holo and apo) and Otrf binding to brucipain (25.0 °C).

pH	bLf	hLf-holo	hLf-apo	Otrf
4.0	$(1.1\pm0.1)\times10^{-1}$	$(9.7\pm0.1)\times10^{-2}$	$(1.2\pm0.1)\times10^{-1}$	$(8.1\pm0.7)\times10^{-1}$
5.0	$(3.5\pm0.4)\times10^{-2}$	$(5.8\pm0.1)\times10^{-2}$	$(7.5\pm0.6)\times10^{-2}$	$(7.9\pm0.7)\times10^{-1}$
6.0	$(8.7\pm0.8)\times10^{-3}$	$(1.8\pm0.1)\times10^{-2}$	$(2.6\pm0.1)\times10^{-2}$	$(6.9\pm0.5)\times10^{-1}$
7.4	$(6.7\pm0.5)\times10^{-3}$	$(1.5\pm0.1)\times10^{-2}$	$(2.2\pm0.1)\times10^{-2}$	$(4.7\pm0.3)\times10^{-1}$
8.0	$(1.2\pm0.1)\times10^{-2}$	$(2.2\pm0.1)\times10^{-2}$	$(3.1\pm0.2)\times10^{-2}$	$(4.5\pm0.3)\times10^{-1}$
9.0	$(3.6\pm0.2)\times10^{-2}$	$(3.2\pm0.2)\times10^{-2}$	$(4.4\pm0.3)\times10^{-2}$	$(4.4\pm0.3)\times10^{-1}$

For all the proteases considered in this study, the dependence of the apparent inhibition constant K_{Iapp} on the Z-Phe-Arg-AMC concentration, was linear over the substrate concentration range explored (Fig. 10). These results are compatible only with a complete competitive inhibition mechanism (Scheme III), in fact, the observed relative inhibition constant K_{Iapp}/K_I increases linearly with the substrate concentrations (Fig. 10). Notably, the Y intercept of straight lines is 1. This means that in the absence of the substrate ([Z-Phe-Arg-AMC] = 0, corresponding to the X interpolation value of the Y intercept) the apparent inhibition constant K_{Iapp}

corrisponds to the intrinsic ligand-independent equilibrium constant K_I (K_{Iapp}= K_I).

Moreover, if we compare values the intrinsic substrate-independent constant K_I with those cystatins, it appears that Lf a very good inhibitor of cisteine papain-like proteases (Table 9).

Table 9. Values of the intrinsic ligand-independent constant K_I (μM) of stefin A, of stefin B (cystatins of type I), of chicken egg-white cystatin (CEW Cys, cystatin of type II).

Proteases	Papain	Cathepsin-B	Cathepsin-L	Cruzipain
Stefin A	1.8×10^{-7} [a]	9.1×10^{-4} [a]	1.9×10^{-5} [a]	2.1×10^{-5} [c]
Stefin B	4.9×10^{-8} [b]	1.8×10^{-2} [b]	3.0×10^{-6} [b]	6.0×10^{-5} [c]
CEW Cys	1.0×10^{-5} [d]	8.0×10^{-5} [d]	3.0×10^{-6} [d]	1.4×10^{-5} [c]

[a] Estrada *et al*. (1998).

[b] Pol & Björk (2001).

[c] Stoka *et al*. (1995).

[d] Anastasi *et al*. (1983).

3.2.2. Effect of pH on values of K_I

The analysis of the pH dependence of the intrinsic ligand-independent inhibition constant K_I (Fig. 11, 12, 13 and 14), according to Equations (9) and (10) allowed the evaluation of pK_a values that define the acid-base equilibrium of amino acidic residue(s) able to modulate the enzyme(s)-Transferrin recognition event (Tables 10, 11, 12 and 13).

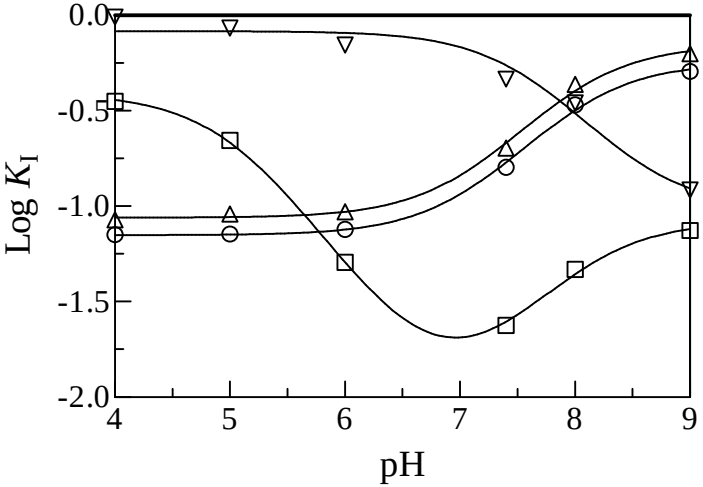

Figure 11: pH dependence of K_I (μM) for the binding of bLf (squares), hLf-holo and -apo (triangles and circles, respectively) and Otrf (reversed triangles) to papain at 25 °C. Solid lines representing the best fit of data, were calculated according to Equation (9) for Otrf, Equation (10) for hLf (apo and holo) and equation (11) for bLf. Continous lines were obtained through an iterative non-linear least squares procedure with sets of parameters given in Tables 5, 6, 7 and 8. For further details see the text.

Table 10: Values of pK_a modulating the inhibition constant K_I (μM) for bLf (squares), hLf-apo and –holo (circles and triangles) and Otrf (overturned triangles) to papain (Fig. 6) at 25 °C.

	pK_a Values	
bLf	pK'_{unl} = 6.9±0.3	pK'_{lig} = 5.0±0.2
	pK''_{unl} = 7.9±0,1	pK''_{lig} = 8.5±0,1
hLf-apo	pK'_{unl} = 7.1±0.3	pK'_{lig} = 8.0±0.2
hLf-holo	pK'_{unl} = 7.1±0.3	pK'_{lig} = 8.0±0.2
Otrf	pK'_{unl} = 8.2±0.2	pK'_{lig} = 7.3±0.2

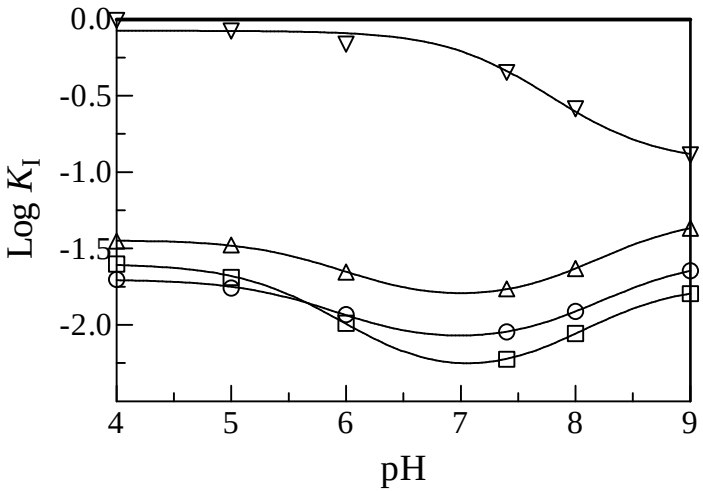

Figure 12: pH dependence of K_I (μM) for the binding of bLf (squares), hLf-holo and -apo (triangles and circles, respectively) and Otrf (reversed triangles) to *L. infantum* protease at 25 °C. Solid lines representing the best fit of data, were calculated according to Equation (9) for Otrf and according to equation (11) for bLf and for hLf (apo and holo). Continous lines were obtained through an iterative non-linear least squares procedure with sets of parameters given in Tables 5, 6, 7 and 8. For further details see the text.

Table 11: Values of pK_a modulating the inhibition constant K_I (μM) regarding for bLf (squares), hLf-apo and –holo (circles and triangles) and Otrf (overturned triangles) to *L. infantum* protease (Fig. 7) at 25 °C.

	pK_a Values	
bLf	pK'$_{unl}$ = 6.4±0.3	pK'$_{lig}$ = 5.6±0.1
	pK"$_{unl}$ = 7.7±0.2	pK"$_{lig}$ = 8.3±0.4
hLf-apo	pK'$_{unl}$ = 6.2±0.5	pK'$_{lig}$ = 5.8±0.2
	pK"$_{unl}$ = 7.9±0.2	pK"$_{lig}$ = 8.5±0.3
hLf-holo	pK'$_{unl}$ = 6.2±0.5	pK'$_{lig}$ = 5.8±0.2
	pK"$_{unl}$ = 7.9±0.2	pK"$_{lig}$ = 8.5±0.3
Otrf	pK'$_{unl}$ = 8.5±0.2	pK'$_{lig}$ = 7.5±0.3

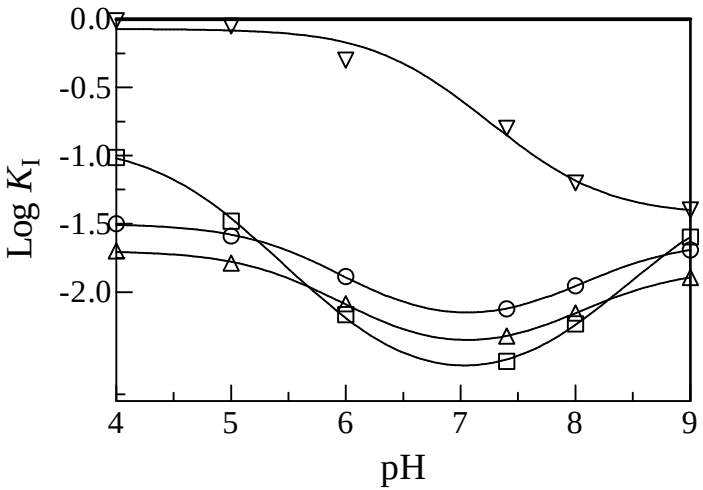

Figure 13: pH dependence of K_I (μM) for the binding of bLf (squares), hLf-holo and -apo (triangles and circles, respectively) and Otrf (reversed triangles) to cruzipain at 25 °C. Solid lines representing the best fit of data, were calculated according to Equation (9) for Otrf and according to equation (11) for bLf and for hLf (apo and holo). Continous lines were obtained through an iterative non-linear least squares procedure with sets of parameters given in Tables 5, 6, 7 and 8. For further details see the text.

Table 12: Values of pK_a modulating the inhibition constant K_I (μM) for bLf (squares), hLf-apo and –holo (circles and triangles) and Otrf (overturned triangles) to cruzipain (Fig. 8) at 25 °C.

	pK_a Values	
bLf	pK'_{unl} = 6.4±0.2	pK'_{lig} = 4.6±0.1
	pK''_{unl} = 7.7±0.2	pK''_{lig} = 9.1±0.3
hLf-apo	pK'_{unl} = 6.4±0.1	pK'_{lig} = 5.6±0.3
	pK''_{unl} = 7.7±0.1	pK''_{lig} = 8.3±0.2
hLf-holo	pK'_{unl} = 6.4±0.1	pK'_{lig} = 5.6±0.3
	pK''_{unl} = 7.7±0.1	pK''_{lig} = 8.3±0.2
Otrf	pK'_{unl} = 7.9±0.2	pK'_{lig} = 6.6±0.3

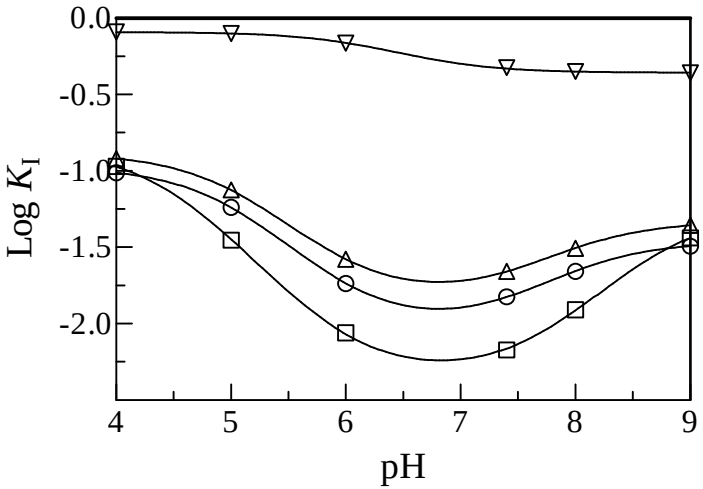

Figure 14: pH dependence of K_I (μM) for the binding of bLf (squares), hLf-holo and -apo (triangles and circles, respectively) and Otrf (reversed triangles) to brucipain at 25 °C. Solid lines representing the best fit of data, were calculated according to Equation (9) for Otrf and according to equation (11) for bLf and for hLf (apo and holo). Continous lines were obtained through an iterative non-linear least squares procedure with sets of parameters given in Tables 5, 6, 7 and 8. For further details see the text.

Table 13: Values of pK_a modulating the inhibition constant K_I (μM) for bLf (squares), hLf-apo and –holo (circles and triangles) and Otrf (overturned triangles) to cruzipain (Fig. 9) at 25 °C.

	pK_a Values	
bLf	pK'_{unl} = 6.2±0.2	pK'_{lig} = 4.3±0.1
	pK''_{unl} = 7.5±0.2	pK''_{lig} = 8.9±0.3
hLf-apo	pK'_{unl} = 6.1±0.2	pK'_{lig} = 5.0±0.1
	pK''_{unl} = 7.4±0.2	pK''_{lig} = 8.0±0.3
hLf-holo	pK'_{unl} = 6.1±0.2	pK'_{lig} = 5.0±0.1
	pK''_{unl} = 7.4±0.2	pK''_{lig} = 8.0±0.3
Otrf	pK'_{unl} = 7.1±0.1	pK'_{lig} = 5.9±0.2

In particular, bLf binding to papain (pK'_{unl} = 6.9±0.3, pK'_{lig} = 5.0±0.2, pK''_{unl} = 7.9±0,1 and pK''_{lig} = 8.5±0,1), *L.infantum* protease (pK'_{unl} = 6.4±0.3, pK'_{lig} = 5.6±0.1, pK''_{unl} = 7.7±0.2 and pK''_{lig} = 8.3±0.4), cruzipain (pK'_{unl} = 6.4±0.2, pK'_{lig} = 4.6±0.1, pK''_{unl} = 7.7±0.2 and pK''_{lig} = 9.1±0.3) and brucipain (pK'_{unl} = 6.2±0.2, pK'_{lig} = 4.3±0.1, pK''_{unl} = 7.5±0.2 and pK''_{lig} = 8.9±0.3) is modulated by the acid-base equilibrium of two amino acidic residues (Fig. 6-9 and Tables 9-12). Inhibitor binding induces a pK_a shift of about 1.9 pH units toward acidic values and of about 0.6 pH units toward the alkaline region for papain ($pK^*_{unl} > pK^*_{lig}$ and $pK^{**}_{unl} < pK^{**}_{lig}$), a pK_a shift of about 0.8 pH units toward acidic values and of about 0.6 pH units toward the alkaline region for protease from *L. Infantum*, a pK_a shift of about 1.8 pH units toward acidic values and of about 1.4 pH units toward the alkaline region for cruzipain and a pK_a shift of about 1.9 pH units toward acidic values and of about 1.4 pH units toward the alkaline region for brucipain.

The interaction of hLf (apo and holo) with papain is modulated by one apparent acidbase ionization (Fig. 6-9 and Tables 9-12) causing a pK_a shift of about 0.9 pH units toward alkaline values (pK'_{unl} = 7.1±0.3 and pK'_{lig} = 8.0±0.2, both apo-form and holo-form). On the other hand, hLf (apo and holo) binding to parasitic proteases is modulated by the acid-base equilibrium of two amino acid residues (Fig. 6-9 and Tables 9-12) causing a pK_a shift of about 0.4 pH units toward acidic values and of about 0.6 pH units toward the alkaline region for *L. Infantum* protease (pK'_{unl} = 6.2±0.5, pK'_{lig} = 5.8±0.2, pK''_{unl} = 7.9±0.2 and pK''_{lig} = 8.5±0.3), a pK_a shift of about 0.8 pH units toward acidic values and of about 0.6 pH units toward the alkaline region for cruzipain (pK'_{unl} = 6.4±0.1, pK'_{lig} = 5.6±0.3, pK''_{unl} = 7.7±0.1 and pK''_{lig} = 8.3±0.2) and a pK_a shift of about 1.1 pH units toward acidic values and of about 0.6 pH units toward the alkaline region for brucipain (pK'_{unl} = 6.1±0.2, pK'_{lig} = 5.0±0.1, pK''_{unl} = 7.4±0.2 and pK''_{lig} = 8.0±0.3).

Instead, the binding of Otrf bLf to papain (pK'_{unl} = 8.2±0.2 and pK'_{lig} = 7.3±0.2), to protease of type I from *L. infantum* (pK'_{unl} = 8.5±0.2 and pK'_{lig} = 7.5±0.3), to cruzipain (pK'_{unl} = 7.9±0.2 and pK'_{lig} = 6.6±0.3) and to brucipain (pK'_{unl} = 7.1±0.1 and pK'_{lig} = 5.9±0.2) is modulated by one apparent amino acidic residue (Fig. 6-9 and Tables 9-12). It causes a pK_a shift of about 0.9 pH units for papain, 1.0 pH units for protease of type I from *L. infantum*, 1.3 pH units for cruzipain and 1.2 pH units for brucipain toward acidic values.

Tfs inhibit preferentially parasitic proteases; the pH optimum is around neutrality for bLf, while it is acidic for hLf and alkaline for Otrf. On the contrary, sTf does not inhibit any enzyme, according to its different role in mammals. In fact sTf function is to transport iron in blood serum. Note that values of K_I are independent of iron saturation (Tables 8-12).

According to 'linked functions' (Wyman, 1964) the (de)protonation of amino acidic residue involved in the ligand binding lead to the modulation of the binding itself in such a way that the effect of pH on the thermodynamic constant(s) (K_m, K_i and K_I) is quantitatively the same of the effect on the pK_a shift (regarding to the acid-base equilibrium of the modulating residues of the protein) induced by the binding of the ligand ($\Delta pK_a = \Delta pK_m$, $\Delta pK_a = \Delta pK_i$ or $\Delta pK_a = \Delta pK_I$, depending on the ligand) (Wyman, 1968). Therefore, the higher pK_a values observed in the binding of inhibitors respect with the synthetic substrate Z-Phe-Arg-AMC probably indicates a much larger free energy variation upon ligand binding, reflecting a wider surface contacting protease-inhibitor complex formation (Wyman, 1964).

Macromolecular recognition occuring between the active site of the cysteine proteases studied and the (macro)molecular species (regardless if they act as inhibitors or substrates) is defined by a thermodynamic constant K (K_m, K_i and K_I, as previously seen) which represents the all-in affinity that exists between two macromolecular species (i.e. the enzyme and its ligand). Therefore, a K constant must be seen as the result of the sum of all the thermodynamic contribution that came from each enzyme-ligand interacting residues.

Accordingly, the calculated pK_a must to be intended as apparent: namely not necessary attributable to the acid-base equilibrium of one amino acidic residue, but rather as a mean value related to the acid-base equilibrium constant of all the ionizing residues which contribute energetically to the enzyme/ligand recognition event.

3.3. Inhibition mechanism of Lactoferrin

As shown in Fig. 15 Lf and Otrf are easily degraded by papain and by parasite cysteine proteases during the assay incubation time.

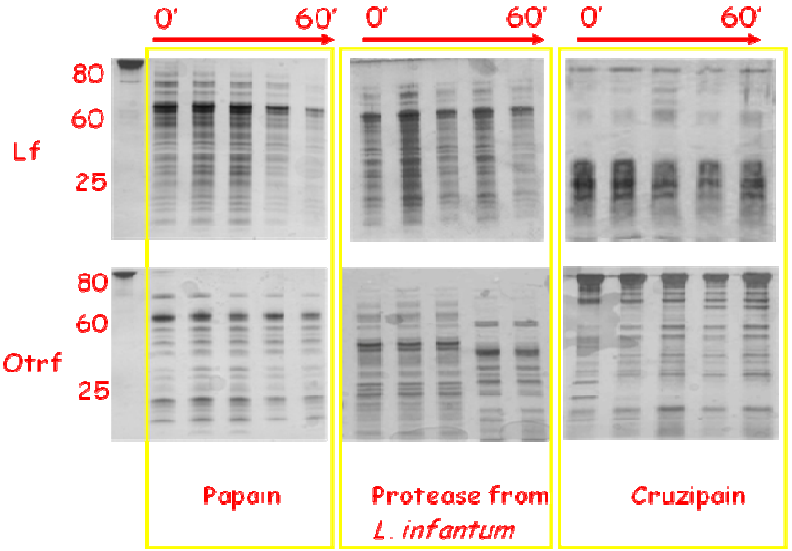

Figure 15: SDS-PAGE showing that Lf is easily degraded by cysteine proteases during the assay incubation time. *Line 1*: Lf or Otrf. *Lines 2,3,4, 5 and 6*: Lf/Otrf and cysteine proteases at times 0, 1, 10, 30 and 60 min. SDS-PAGE was obtained at 12% polyacrylamide

Analysis by SDS–PAGE revealed different peptide profiles from Lf hydrolysis (Fig. 15). The incubation time also influenced the degradation extent of Lf. In fact papain and the parasite cysteine proteases generate peptides of range between 35 kDa and 10 kDa. The maximum hydrolysis values (100%) were reached after 30 minutes of incubation for all studied enzymes.When the incubation period was prolonged up to 24 h, smaller size peptides are detected for all strains.

In presence of antipain, an inhibitor of papain-like proteases, Lf and Otrf are not degraded (Fig 16).

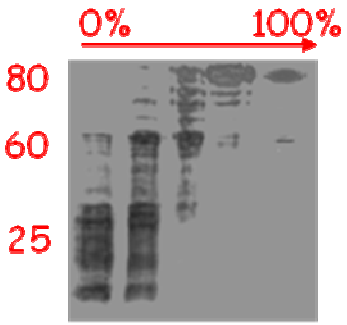

Figure 16: Effect of antipain on Lf hydrolysis by papain. Line 1 absence of antipain. Line 2, 3, 4 and 5 increased concentration of antipain (0.25:1; 0.50:1; 0.75:1 and 1:1 antipain:papain)

Since the Lf sequence 679-695 is similar to the cystatin active site, it is possible that a peptidic cystatin-like inhibitor is generated by enzymes-induced Lf hydrolysis.

3.4. MALDI TOF/TOF analysis

In order to characterize the peptides produced by bLf and hLf papain digestions, a mass spectrometry (MS) analysis was performed. To this end, each inhibitor digestion was analyzed by peptide mass fingerprinting and identified peptides are listed in Tables 14 and 15.

In bLf MS analysis we covered the 60% of protein sequence by detection of 57 peptides.

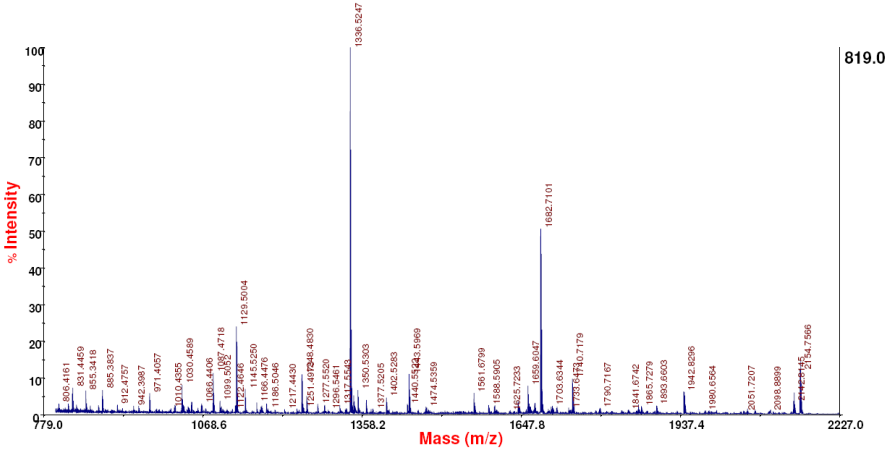

Figure 17. MS spectrum of bLf digestion by papain

Table 14:MS analysis of peptides produced by bLf papain digestion

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence
830.473	830.4823	0.0093	11	596	602	TRKPVTE
831.4505	831.4459	-0.0046	-6	516	522	PKSRLCA
855.3916	855.3505	-0.0411	-48	77	84	TLDGGMVF
885.3988	885.3831	-0.0157	-18	154	160	YLSWTES
971.4356	971.4045	-0.0311	-32	674	681	PTYEEYLG
1030.4623	1030.4563	-0.006	-6	508	517	QSCAPGADPK
1030.5503	1030.4563	-0.094	-91	140	148	RSAGWIIPM
1065.463	1065.4208	-0.0422	-40	383	392	QSGQNVTCAT
1087.495	1087.4711	-0.0239	-22	476	485	CHTAVDRTAG
1087.5717	1087.4711	-0.1006	-92	140	149	RSAGWIIPMG
1099.5643	1099.5044	-0.0599	-54	632	641	HQQALFGKNG
1127.5255	1127.4244	-0.1011	-90	154	162	YLSWTESLE
1129.4619	1129.5	0.0381	34	504	513	EFFSQSCAPG
1129.6	1129.5	-0.1	-89	310	319	SPPGQRDLLF
1145.5255	1145.5232	-0.0023	-2	528	537	DQGLDKCVPN
1145.5983	1145.5232	-0.0751	-66	61	70	ALECIRAIAE
1166.4459	1166.4457	-0.0002	0	498	507	GSCAFDEFFS
1184.5404	1184.4501	-0.0903	-76	82	91	MVFEAGRDPY
1248.579	1248.4829	-0.0961	-77	263	273	ECHLAQVPSHA
1248.667	1248.4829	-0.1841	-147	41	49	WQWRMKKLG
1257.5569	1257.5709	0.014	11	81	91	GMVFEAGRDPY
1277.5757	1277.549	-0.0267	-21	563	573	KNDTVWENTNG
1289.5869	1289.5516	-0.0353	-27	378	388	QQWSQQSGQNV
1317.574	1317.5579	-0.0161	-12	526	537	GDDQGLDKCVPN
1336.5626	1336.5247	-0.0379	-28	201	211	ACSSKEPYFGY
1336.7083	1336.5247	-0.1836	-137	148	158	MGILRPYLSWT
1342.6273	1342.5541	-0.0732	-55	671	682	GGRPTYEEYLGT
1350.5492	1350.5303	-0.0189	-14	377	387	CQQWSQQSGQN
1350.6624	1350.5303	-0.1321	-98	30	39	ISQPEWFKCR
1365.5852	1365.5714	-0.0138	-10	380	391	WSQQSGQNVTCA
1402.6566	1402.5271	-0.1295	-92	510	522	CAPGADPKSRLCA
1440.5907	1440.5513	-0.0394	-27	385	398	GQNVTCATASTTDD
1443.6759	1443.5966	-0.0793	-55	167	179	AVAKFFSASCVPC
1443.7234	1443.5966	-0.1268	-88	129	141	QGRKSCHTGLGRS
1474.6492	1474.5311	-0.1181	-80	475	487	SCHTAVDRTAGWN
1561.7097	1561.6774	-0.0323	-21	367	379	CAVGPEEQKKCQQ
1561.8333	1561.6774	-0.1559	-100	117	130	VKKGSNFQLDQLQG
1598.7697	1598.5991	-0.1706	-107	405	419	KGEADALNLDGGYIY
1641.7189	1641.5986	-0.1203	-73	27	38	WCTISQPEWFKC
1659.7869	1659.6031	-0.1838	-111	644	656	CPDKFCLFKSETK
1659.89	1659.6031	-0.2869	-173	40	52	RWQWRMKKLGAPS
1663.7891	1663.6001	-0.189	-114	517	531	KSRLCALCAGDDQGL
1671.7869	1671.7449	-0.042	-25	167	181	AVAKFFSASCVPCID
1682.8748	1682.7075	-0.1673	-99	650	663	LFKSETKNLLFNDN
1701.9258	1701.6786	-0.2472	-145	41	54	WQWRMKKLGAPSIT
1733.8016	1733.6287	-0.1729	-100	671	685	GGRPTYEEYLGTEYV
1740.7686	1740.7174	-0.0512	-29	208	223	YFGYSGAFKCLQDGAG
1740.881	1740.7174	-0.1636	-94	124	138	QLDQLQGRKSCHTGL
1789.8385	1789.7494	-0.0891	-50	526	541	GDDQGLDKCVPNSKEK
1859.8705	1859.6991	-0.1714	-92	369	383	VGPEEQKKCQQWSQQ
1865.8665	1865.7321	-0.1344	-72	557	573	GDVAFVKNDTVWENTNG
1893.8405	1893.6523	-0.1882	-99	176	190	CVPCIDRQAYPNLCQ
1942.8639	1942.8282	-0.0357	-18	540	555	EKYYGYTGAFRCLAED
1942.9803	1942.8282	-0.1521	-78	481	498	DRTAGWNIPMGLIVNQTG
2143.1843	2142.8123	-0.372	-174	617	635	VSRSDRAAHVKQVLLHQQA
2155.0488	2154.7578	-0.291	-135	549	567	FRCLAEDVGDVAFVKNDTV
2155.238	2154.7578	-0.4802	-223	1	21	MKLFVPALLSLGALGLCLAAP

Particularly, we were able to detect a signal at 1733.63 *m/z* which corresponds to residues 671–685 (GGRPTYEEYLGTEYV) of the bLf protein (Fig 18). Interestingly, this peptide was found eluited in HPLC fraction able to inhibit the cysteine papain-like proteases in dose dependent way and shows in the C-terminal region a cystatine active site sequence (Table 1).

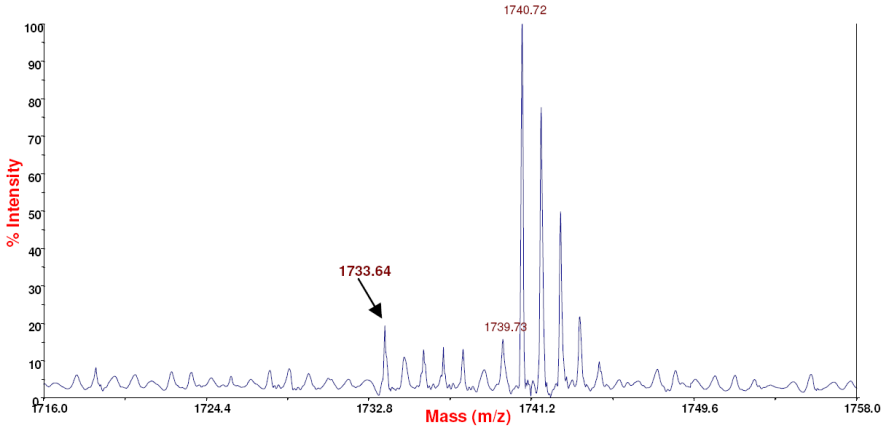

Figure18. MS spectrum of bLf digestion by papain. The image shows the zoom range of 1733.64 peak MS/MS spectrum

In hLf MS analysis we covered more of 60% of sequence (72 peptides matched) (Table 15) detecting two signals (i.e.: 1266.69 and 1646.84 *m/z*) corresponding to residues YVAGITNLKKC (687-697) and GKTTYEKYLGPQYV (675-688) respectively (Fig. 19 and 20). Both of them were found in two different HPLC fractions with inhibitor activity and show a portion of cystatin-like sequence.

Table 15: MS analysis of peptides produced by hLf papain digestion

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence
806.4042	806.4178	0.0136	17	467	473	SLTWNSV
824.3937	824.4005	0.0068	8	110	116	THYYAVA
856.4199	856.3755	-0.0444	-52	344	350	YFTAIQN
884.5649	884.5267	-0.0382	-43	18	24	LAGRRRR
885.5305	885.5516	0.0211	24	286	292	AIWNLLR
885.5516	885.5516	0	0	627	633	ERLKQVL
942.5743	942.5783	0.004	4	361	367	RRARVVW
970.6155	970.6053	-0.0102	-11	456	464	LAVAVVRRS
998.5305	998.5516	0.0211	21	228	235	FIRESTVF
1013.6141	1013.6218	0.0077	8	148	156	PIGTLRPFL
1052.5735	1052.5818	0.0083	8	162	171	PEPIEAAVAR
1070.5887	1070.6075	0.0188	18	45	53	NMRKVRGPP
1102.564	1102.5671	0.0031	3	482	491	AVDRTAGWNI
1102.5891	1102.5671	-0.022	-20	163	172	EPIEAAVARF
1222.6136	1222.6838	0.0702	57	589	599	ADFALLCLDGK
1229.6888	1229.6979	0.0091	7	330	340	VPPRIDSGLYL
1256.6892	1256.6813	-0.0079	-6	45	55	NMRKVRGPPVS
1266.6875	1266.6865	-0.001	-1	687	697	YVAGITNLKKC
1268.7256	1268.7184	-0.0072	-6	47	57	RKVRGPPVSCI
1268.7256	1268.7184	-0.0072	-6	49	59	VRGPPVSCIKR
1284.6841	1284.6785	-0.0056	-4	50	60	RGPPVSCIKRD
1314.7164	1314.7336	0.0172	13	282	292	GKEDAIWNLLR
1321.7335	1321.7657	0.0322	24	271	283	PSHAVVARSVNGK
1336.6896	1336.6663	-0.0233	-17	163	174	EPIEAAVARFFS
1352.6263	1352.6447	0.0184	14	662	672	LFNDNTECLAR
1407.7413	1407.7531	0.0118	8	468	479	LTWNSVKGKKSC
1414.6783	1414.7056	0.0273	19	166	178	EAAVARFFSASCV
1424.6176	1424.8143	0.1967	138	234	245	VFEDLSDEAERD
1424.8121	1424.8143	0.0022	2	631	642	QVLLHQQAKFGR
1431.7162	1431.7296	0.0134	9	470	481	WNSVKGKKSCHT
1513.7533	1513.7616	0.0083	5	227	239	AFIRESTVFEDLS
1558.7972	1558.7997	0.0025	2	276	289	VARSVNGKEDAIWN
1579.7533	1579.7644	0.0111	7	660	672	NLLFNDNTECLAR
1596.8744	1596.8667	-0.0077	-5	316	329	KDLLFKDSAIGFSR
1614.8597	1614.8693	0.0096	6	279	292	SVNGKEDAIWNLLR
1646.8424	1646.8436	0.0012	1	675	688	GKTTYEKYLGPQYV
1649.7999	1649.8685	0.0686	42	608	622	SCHLAMAPNHAVVSR
1658.8707	1658.881	0.0103	6	346	360	TAIQNLRKSEEEVAA
1661.8315	1661.8092	-0.0223	-13	446	460	NCVDRPVEGYLAVAV
1666.95	1666.923	-0.027	-16	630	643	KQVLLHQQAKFGRN
1701.79	1701.8104	0.0204	12	443	457	PDPNCVDRPVEGYLA
1718.8101	1718.8162	0.0061	4	177	191	CVPGADKGQFPNLCR
1752.9789	1752.9333	-0.0456	-26	623	636	MDKVERLKQVLLHQ
1753.9126	1753.9204	0.0078	4	597	611	DGKRKPVTEARSCHL
1770.9609	1770.9664	0.0055	3	278	292	RSVNGKEDAIWNLLR
1805.8422	1805.8531	0.0109	6	176	191	SCVPGADKGQFPNLCR
1808.8894	1808.877	-0.0124	-7	610	625	HLAMAPNHAVVSRMDK
1819.8928	1819.8632	-0.0296	-16	400	417	CIALVLKGEADAMSLDGG
1825.7974	1825.8082	0.0108	6	236	250	EDLSDEAERDEYELL
1854.8551	1854.8459	-0.0092	-5	636	651	QQAKFGRNGSDCPDKF
1863.9004	1863.9215	0.0211	11	394	411	ASTTEDCIALVLKGEADA
1876.8793	1876.8782	-0.0011	-1	366	380	VWCAVGEQELRKCNQ
1903.8491	1903.8456	-0.0035	-2	441	457	SDPDPNCVDRPVEGYLA

1904.0276	1903.8456	-0.182	-96	86	103	EAGLAPYKLRPVAAEVYG
1963.9066	1963.9056	-0.001	-1	245	260	DEYELLCPDNTRKPVD
2024.9706	2024.9531	-0.0175	-9	656	672	SETKNLLFNDNTECLAR
2103.948	2103.948	0	0	200	217	KCAFSSQEPYFSYSGAFK
2168.0098	2168.0295	0.0197	9	396	415	TTEDCIALVLKGEADAMSLD
2172.9583	2172.9558	-0.0025	-1	188	206	NLCRLCAGTGENKCAFSSQ
2371.1321	2371.0325	-0.0996	-42	172	192	FFSASCVPGADKGQFPNLCRL
2389.0564	2389.0173	-0.0391	-16	231	250	ESTVFEDLSDEAERDEYELL
2528.0894	2528.0591	-0.0303	-12	540	561	NSNERYYGYTGAFRCLAENAGD
2545.0923	2545.0647	-0.0276	-11	233	253	TVFEDLSDEAERDEYELLCPD
2545.1082	2545.0647	-0.0435	-17	526	546	LCIGDEQGENKCVPNSNERYY
2561.1936	2561.0828	-0.1108	-43	436	458	KSQQSSDPDPNCVDRPVEGYLAV
2577.1172	2577.1265	0.0093	4	201	222	CAFSSQEPYFSYSGAFKCLRDG
2602.1296	2602.0645	-0.0651	-25	526	547	LCIGDEQGENKCVPNSNERYYG
2770.2097	2770.2407	0.031	11	532	554	QGENKCVPNSNERYYGYTGAFRC
2787.2136	2787.2183	0.0047	2	191	215	RLCAGTGENKCAFSSQEPYFSYSGA
2787.4617	2787.2183	-0.2434	-87	151	175	TLRPFLNWTGPPEPIEAAVARFFSA
2805.3101	2805.2236	-0.0865	-31	228	250	FIRESTVFEDLSDEAERDEYELL
3037.4016	3037.3098	-0.0918	-30	394	422	ASTTEDCIALVLKGEADAMSLDGGYVYTA

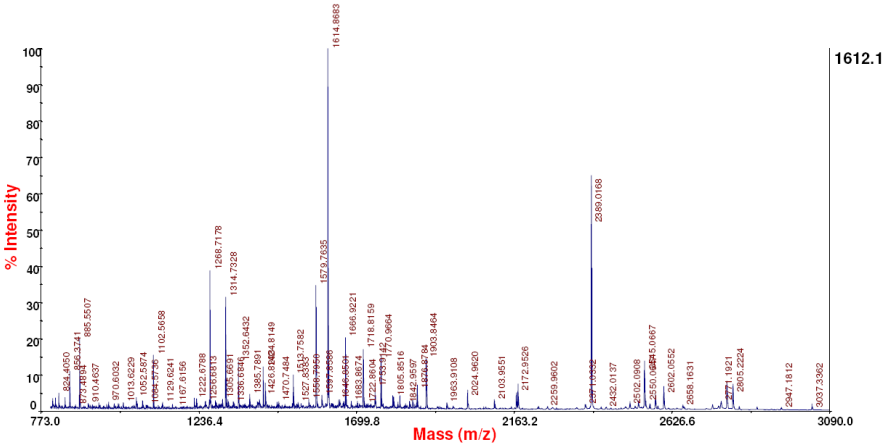

Figure18. MS spectrum of hLf digestion by papain

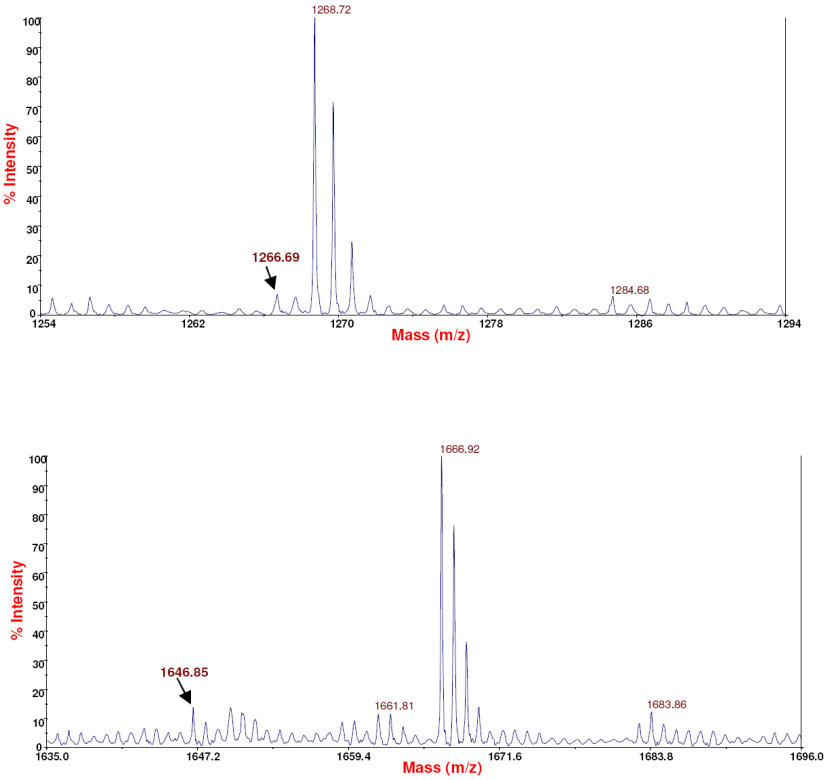

Figure 19: MS spectrum of hLf digestion by papain. The image shows the zoom range of 1266.69 and 1646.84 peaks MS/MS spectrum

These data suggest that these peptides generated from papain hydrolysis could inhibit the protease itself.

3.5. Molecular modelling of putative complex Y687-G690-Papain

The sequences of hypotetical inhibitor fragments are aligned (Table 16) using ClustalW alignament software.

Table 16: Alignment of inhibitor peptides with cystatine active site

cystatine active site	LG QVVAG
hLf	YVAGITNLKKC
hLf	GKTTYEKYLGPQYV
bLf	GGRPTYEEYLGTEYV

In fig. 20 the result of this procedue is showed. The "YV" motiv seems to be conserved in all fragment tested. On the basis of the proposed alignments a structural preliminary model is done to test the compatibility of these residues with the mechanism of catalysis. To this aim, a site-specific mutation was performed *in silico* through the utilization of "Swiss pdb viewer" (Guex, N. & Peitsch, M.C. 1997) molecular modeling software. The residues VVAG of Stefin B are mutated in YVAG. For each mutation, the software offers, through an assessment of possible interactions with the substrate, the best rotamers (different disposition of the amino acid residues in three-dimentionl space). The crystal structure of the papain-stefin B complex (code PDP: 1STF) is used to model the interaction (Stubbs *et al*, 1990). The very preliminary results show a likely favorable interaction between Tyr present on the putative inhibitory fragments and the residue Gly23 of papain. Further observation are required to identify the exact bond-condition. In other hands, this evidence, if properly confirmed by further experimental analysis, seems to show that the patterns identified can be regarded as foundamental in the competitive inhibition process.

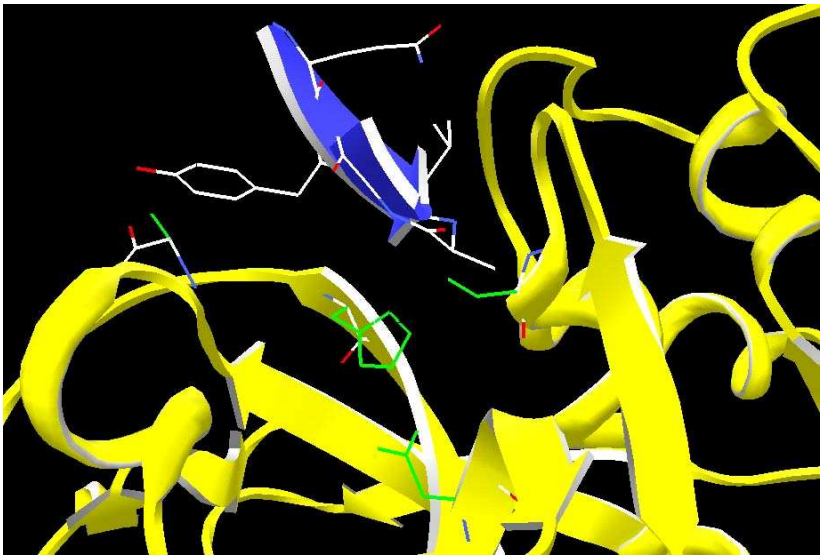

Figure 20: Molecular modelling of putative complex Y687-G690-Papain. Site-specific mutation was performed *in silico* through the utilization of "Swiss pdb viewer" (Guex, N. & Peitsch, M.C. 1997) molecular modeling software. The crystal structure of the papain-stefin B complex (code PDP: 1STF) is used to model the interaction (Stubbs *et al*, 1990).

Conclusions

In this study we have investigated the thermodynamic parameters of human lactoferrin (hLf), bovine lactoferrin (bLf) and hen's ovotransferrin (Otrf) inhibition of the parasitic papain-like type I cysteine proteases from *Leishmania infantum*, *Trypanosoma cruzii* and *Trypanosoma brucei*.

Parasites synthesize papain-like cysteine proteases that are relevant for the virulence and pathogenicity of parasites, being involved in several aspects of the parasite life cycle, it is therefore possible that the antiparasitic activity of lactoferrin could be due to the inhibition of parasitic papain-like cysteine protease that we have recently observed.

Transferrins belong to a family of iron-binding glycoproteins possessing similar aminoacid sequence though they have different biological functions and locations. Lactoferrin is expressed and secreted from glandular epithelial cells and from mature neutrophiles of mammalian and it is an important component of the aspecific host defence or natural immunity, including resistance to parasitic infections. Serum transferrin is synthesized by the liver of mammals and secreted into the blood stream; its primary function is iron transport. Ovotransferrin, synthesized by avians, displays both iron transport and protective functions.

bLf, hLf and Otrf, both in the apo- and olo-forms, showed time- and concentration-dependent inhibition of the catalytic activity of papain and of type I proteases from *L.infantum*, *T. cruzii* and *T. brucei*. The K_I values observed for bLf and hLf inhibition of *L. infantum*, *T. cruzi* and *T. brucei* proteases were in the nanomolar range (K_I = 3.1 nM), lower than K_I values observed for papain inhibition (K_I = 24 nM). Otrf showed lower inhibition of cysteine proteases (K_I = 0.6 μM). On the contrary, serum transferrin did not display any inhibition towards type I parasitic proteases, according to its different role in mammals. The inhibition of type I parasitic cysteine proteases by hLf, bLf and Otrf appeared to conform to a competitive mechanism. The observed pH optimum for bLf inhibition of parasitic proteases was around neutrality, while it was acidic for hLf and alkaline for Otrf. The further quantitative analysis of the pH dependence of the intrinsic ligand-independent inhibition constant K_I allowed the evaluation of pK_a values that define the acid-base equilibrium of amino acidic residue(s) modulating the enzyme(s)-inhibitor recognition events. SDS-PAGE showed that hLf, bLf and Otrf were easily degraded by either

papain or parasitic type I protease during the assay incubation time (few minutes) and it is likely that one or more protease inhibitory peptides were generated from protein hydrolysis.

As a matter of fact, a sequence present near the C-terminus of human (hLf) and bovine (bLf) lactoferrin shows homology with the sequence of the active site of cystatins, which are competitive inhibitors of papain-like cysteine proteases. The same sequence is present, though with lower homology, in Otrf and, with even lower homology, in sTf. Therefore, we have studied by MALDI-TOF the profile of Lf cleavage by papain and preliminary data suggest the presence of a cystatin-like peptide in two proteolytic fragments of hLf and in one proteolytic fragment of bLf. The work will continue with the characterization of the active peptides looking at the possible design of a peptidomimetic drug for the therapy of parasitic infections.

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Materials and Methods

Materials

Enzymes: papain was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cruzipain was prepared from *T. cruzi* (CAN-III strain) epimastigotes (Lima *et al*., 1992; Labriola *et al*., 1993; Stoka *et al*., 1995; Salvati *et al*., 2001a). Brucipain was prepared from *T. Brucei* (MBOS/NG/94/NITR strain) (Lima *et al*., 1992; Labriola *et al*., 1993; Stoka *et al*., 1995; Salvati *et al*., 2001a). The *L. infantum* cysteine protease was prepared from parasite (strain MHOM/TN/80/IPT1) promastigotes (Labriola *et al*., 1993; Selzer *et al*., 1997; Salvati *et al*., 2001b). The reducing agent dithiothreitol does not significantly activate (<10%) the freshly prepared cruzipain and the *L. infantum* cysteine protease (Salvati *et al*., 2001a, 2001b; Venturini *et al*., 2000). The concentration of active cruzipain, brucipain, *L. infantum* cysteine proteases and papain was determined by active site titration with antipain (Barrett & Salvesen, 1986; Rosenthal *et al*., 1989).

Inhibitors: Bovine Lactoferrin (bLf) was extracted and purified from raw cow milk according to Sharma *et al.* (1999). Human Lactoferrin (hLf) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Holo- and apo-forms were obtained according to Giansanti *et al.* (2002). Ovotransferrin (Otrf) was purified from chicken egg white, as described previously (Phelps & Antonini, 1975). Serum Transferrin (sTf) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Chemicals: *N*-α-benzyloxycarbonyl-L-phenylalanyl-L-arginine-(7-amino-4-methyl coumarin) (Z-Phe-Arg-AMC), dithiothreitol, dimethylsulfoxide DMSO and antipain were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The degree of purity of proteins used in this study was ≥ 99%. All other chemicals were of analytical grade.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). was performed on a 12% polyacrylamide gel and was stained according to Chevallet *et al*. (2006)

Methods

Cysteine protease assay. The catalytic activity of papain, of cruzipain and of the protease from *L. infantum* was determined following the continuous assays of Barrett (Barrett, 1980), based on the determination of the fluorescence emission due to the proteases catalyzed-hydrolysis of the fluorogenic substrate Z-Phe-Arg-AMC. Briefly, the enzymes were

activated incubating the protein solutions with DTT (final concentration, 1mM). Z-Phe-Arg-AMC (final concentration, 1.0×10^{-7} M to 5.0×10^{-5} M for parasite cysteine proteases and 1×10^{-5}M to 1.5×10^{-4}M for papain) was added to the activated cysteine proteases solution after 40 minutes (final concentration, 2.0×10^{-9} M, 1.0×10^{-7} M and 1×10^{-8} M for papain, cruzipain, brucipain and for protease from *L. infantum*, respectively). The inhibitor (i.e. antipain or Lactoferrin), if present, was added after the first 10 minutes of incubation (Lima *et al*., 1992; Stoka *et al*., 1998; Venturini *et al*., 2000). Fluorescence (380 nm excitation wavelength, and 460 nm emission wavelength) was measured continuously over 10 min using a Jasco FP-6500 fluorimeter (Jasco Corporation, Tokyo, Japan). Under all the experimental conditions, the fluorescence change (i.e. the product formation) was linear over the assay time (10 min). All the experiments was carried out at 25 °C.

The thermodynamic parameters related to the hydrolysis of the substrate (i.e. the Michaelis constant, K_m and the substrate inhibition constant, K_i) and to the inhibition of the Lf (i.e. the inhibition constant, K_I) were determined for papain, for cruzipain, for brucipain and for the protease from *L. infantum* in a pH range spanning from pH 4.0 to pH 9.0 (acetate buffer, from pH 4.0 to 6.0; phosphate buffer, from pH 6.0 to 7.8; borate buffer, from pH 7.8 to 9.0; 0.1 M) at 25 °C. No specific effects were observed using different buffers with overlapping pH values. The amount of Z-Phe-Arg-AMC hydrolysed by cysteine proteases was calibrated at every pH and temperature, letting the enzymatic reaction go to completion and measuring the amplitude of the signal.

The hydrolysis of the Z-Phe-Arg-AMC catalyzed by papain was analyzed (Hollaway *et al*., 1971; Antonini & Ascenzi, 1981) in the framework of the minimum mechanism described by Scheme I:

$$\mathrm{E} + \mathrm{S} \underset{}{\overset{K_m}{\leftrightarrow}} \mathrm{EX} \overset{V_{max}}{\rightarrow} \mathrm{E} + \mathrm{P} \qquad \text{(Scheme I)}$$

where E is the papain, S is the fluorogenic substrate Z-Phe-Arg-AMC, EX indicate the catalytic intermediate(s) and P are the hydrolysis products, namely *N*-α-benzyloxycarbonyl-L-phenylalanyl-L-arginine (Z-Phe-Arg) and 7-amino-4-methyl coumarin (AMC). Moreover, K_m is the Michaelis constant and V_{max} (= $k_{cat} \times [E]$) denotes the maximum hydrolysis velocity for each enzyme (that depend on the product of the enzyme concentration [E] with the catalytic constant k_{cat}). The analysis of the thermodynamic parameter K_m related to the papain catalyzed-hydrolysis of the substrate was

performed using the classical relative (relative initial velocity: v_i/V_{max}) of the Michaelis-Menten equation under pseudo first-order conditions (i.e.: [E] < 5 × [S]) according to equation (1):

$$v_i/V_{max} = [S]/(K_m + [S]) \quad (1)$$

The hydrolysis of the Z-Phe-Arg-AMC catalyzed by the cysteine proteases from *T. Cruzi*, *T. brucei* and from *L. infantum* was analyzed according to the minimum mechanism for the total substrate inhibition depicted in Scheme II (Cleland, 1979):

$$\begin{array}{ccccc} & K_m & V_{max} & & \\ E + S_S & \leftrightarrow & EX & \rightarrow & E + P \\ + & & & & \\ S_i & & & & \text{(Scheme II)} \\ \updownarrow & K_i & & & \\ E{:}S_i & & & & \end{array}$$

where E denotes the parasite cysteine proteases, S_S is Z-Phe-Arg-AMC interacting with the enzyme(s) as a substrate, S_i is Z-Phe-Arg-AMC acting as the inhibitor, EX represents the catalytic intermediate(s), P indicates the hydrolysis products Z-Phe-Arg and AMC, $E{:}S_i$ is the reversible enzyme(s):inhibitor complex, K_m is the Michaelis constant for Z-Phe-Arg-AMC as the substrate, V_{max} (= k_{cat} × [E], k_{cat} and [E], representing the catalytic constant and the active enzyme concentration, respectively, as in Scheme I) is the maximum velocity and K_i is the inhibition dissociation equilibrium constant for the binding of Z-Phe-Arg-AMC to enzyme(s) as inhibitor.

The analysis of the thermodynamic parameters (K_m and K_i) related to the cruzipain, the brucipain and to the *L. infantum* cysteine proteases

catalyzed-hydrolysis of the substrate were determined from the dependence of the apparent relative initial velocity (v_i/V_{max}) on the substrate concentration under conditions where [E] < 5 × [S] according to equation (2) (Venturini *et al*., 2000; Stoka *et al*., 2000):

$$v_i/V_{max} = [S]/(K_m + [S] + [S]^2/K_i) \quad (2)$$

The pH dependence of the Michaelis constant (K_m) for the parasite enzymes-catalyzed hydrolysis of the Z-Phe-Arg-AMC was analyzed according to equation (3) (Wyman, 1964; Tipton & Dixon, 1979; Ascenzi *et al*., 1988; Salvati *et al*., 2001a):

$$\mathrm{Log}K_m = \mathrm{Log}K_m^{++} - \mathrm{Log}\{([H^+] + K'_{lig})/([H^+] + K'_{unl})\} \quad (3)$$

where K_m^{++} is the pH-independent acidic value of K_m, K'_{unl} is the apparent acidic dissociation constant referring to the acid-base equilibrium of one amino-acid residue for the Z-Phe-Arg-AMC-free active parasite enzyme(s), and K'_{lig} is the apparent acidic dissociation constant referring to the acid-base equilibrium of the same amino-acid residue for the Z-Phe-Arg-AMC-bound active parasite enzyme(s). Equation (3) accounts for the pK_a shift of one ionizing group of active parasite cysteine proteases upon Z-Phe-Arg-AMC binding (Wyman, 1964; Ascenzi *et al*., 1988; Salvati *et al*., 2001a).

The pH dependence of the thermodynamic parameter K_m for the papain catalyzed-hydrolysis of the Z-Phe-Arg-AMC was analyzed according to equation (4) (Wyman, 1964; Tipton & Dixon, 1979; Ascenzi *et al*., 1988; Salvati *et al*., 2001a):

$$\mathrm{Log}K_m = \mathrm{Log}K_m^{++} - \mathrm{Log}\{([H^+] + K'_{lig})/([H^+] + K'_{unl})\} + \\ + \mathrm{Log}\{([H^+] + K''_{unl})/([H^+] + K''_{lig})\} \quad (4)$$

where K_m^{++} is the pH-independent acidic value of K_m and K'_{lig}, K'_{unl}, K''_{lig} e K''_{unl} are the apparent acidic dissociation constants referring to the acid-base equilibrium of two amino-acid residues (denoted as *K'* for the first and as *K''* for the second residue) for the Z-Phe-Arg-AMC-free (K_{unl}) and for the Z-Phe-Arg-AMC-bound (K_{lig}) active papain, respectively. Therefore, equation (4) accounts for the p*Ka* shift of two ionizing group of active papain upon Z-Phe-Arg-AMC binding (Wyman, 1964; Ascenzi *et al*., 1988; Salvati *et al*., 2001a).

The pH dependence of the thermodynamic parameter K_i which describe the binding equilibrium of the Z-Phe-Arg-AMC, acting as inhibitor, to the parasite cysteine proteases has been analized according to the equation (5) (Wyman, 1964; Tipton & Dixon, 1979; Ascenzi *et al*., 1987; Salvati *et al*., 2001a):

$$\mathrm{Log}K_i = \mathrm{Log}K_i^{++} + \mathrm{Log}\{([H^+] + K°_{unl})/([H^+] + K°_{lig})\} \qquad (5)$$

where K_i^{++} define the pH-independent acidic value of K_i, $K°_{unl}$ and $K°_{lig}$ are the apparent acidic dissociation constants referring to the acid-base equilibrium of one amino-acid residue for the Z-Phe-Arg-AMC-free ($K°_{unl}$) and Z-Phe-Arg-AMC-bound to the parasite cysteine proteases ($K°_{lig}$).

The inhibition of the papain and of the cysteine proteases from *T. cruzi*, *T. Brucei* and from *L. infantum* by the Lf was analyzed in the framework of the minimum mechanism, describing the complete competitive inhibition, depicted in Scheme III:

$$\begin{array}{ccccc} & K_m & V_{max} & & \\ E + S & \leftrightarrow & EX & \rightarrow & E + P \\ + & & & & \\ I & & & & \\ \updownarrow & K_I & & & \\ E{:}I & & & & \end{array} \qquad \text{(Schema III)}$$

Although Scheme III is formally identical to Scheme II, in the former I indicate the proteinaceous inhibitor and K_I refers to the inhibition constant that define the affinity of Lf itself for papain, for cruzipain, for brucipain and for the protease from *L. infantum*. For each protease, the apparent inhibition constant K_{Iapp} related to the enzyme:inhibitor complex was determined according to equation (6), which correlate the dependence of the apparent relative initial velocity $v_i/(v_0 \times V_{max})$ to the inhibitor concentration [I], under pseudo first-order conditions (i.e.: [E] < 5 × [S]) (Belenghi *et al*., 2003):

$$v_i/v_0 = [S]/(K_m \times (1+[I]/K_{Iapp}) + [S]) \quad (6)$$

where v_0 is the initial apparent velocity for the hydrolysis of the substrate (Z-Phe-Arg-AMC) in the absence of inhibitor, v_i denote the initial apparent velocity in the presence of increasing concentrations of inhibitor. The values of K_I obtained with equation (6) must to be intended as apparent values (i.e.: K_{Iapp}) thanks to the competition for free enzyme(s) that occur between the substrate (Z-Phe-Arg-AMC) and the inhibitor (Lf). Therefore, the substrate-independent values of the inhibition constant (K_I) were calculated according to equation (7) which relates K_I to the affinity of each enzyme for the substrate (i.e.: the Michaelis constant K_m) and to the substrate concentration used ([S]) (Serveau *et al*., 1995):

$$K_I = K_{Iapp}/(1+[S]/K_m) \quad (7)$$

The linear equation relative to Equation (7) is defined by Equation (8):

$$K_{Iapp}/K_I = K_m^{-1} \times [S] + 1 \quad (8)$$

Moreover, the pH-dependent interaction of the Transferrin with the papain, with the cruzipain and with the protease from *L. infantum*, described by the inhibition thermodynamic parameter K_I, was analyzed according to

equations (9), (10) and (11), depending on the number of ionizing group(s) which modulate the macromolecular interaction. In particular, the data related to enzyme(s):Otrf complexe, depending on the acid-base equilibrium of only one group (descending curve), have been analyzed according to equation (9) (Ascenzi *et al*., 2004; Bocedi *et al*., 2004):

$$\mathrm{Log}K_I = \mathrm{Log}K_I^{++} - \mathrm{Log}\{([H^+] + K^*_{lig})/([H^+] + K^*_{unl})\} \quad (9)$$

where K_I^{++} is the pH-independent acidic value of K_I, K^*_{lig} and K^*_{unl} are the apparent acidic dissociation constants referring to the acid-base equilibrium of one amino-acid residue for the enzyme(s)-free (K^*_{unl}) and for the enzyme(s):Otrf-bound (K^*_{lig}).

The data related to papain:hLf (apo and holo) complexe, depending on the acid-base equilibrium of only one group (ascending curve), have been analyzed according to equation (10) (Ascenzi *et al*., 2004; Bocedi *et al*., 2004):

$$\mathrm{Log}K_I = \mathrm{Log}K_I^{++} + \mathrm{Log}\{([H^+] + K^{**}_{unl})/([H^+] + K^{**}_{lig})\} \quad (10)$$

where K_I^{++} is the pH-independent acidic value of K_I, K^*_{lig} and K^*_{unl} are the apparent acidic dissociation constants referring to the acid-base equilibrium of one amino-acid residue for the enzyme(s)-free (K^*_{unl}) and for the papaina:hLf (apo and holo)-bound (K^*_{lig}).

The data related to the interaction of the papain with bLf and of the parasitic proteases with bLf, hLf (apo and holo) are analyzed on the basis of the acid-base equilibrium of two ionising groups, according to the equation (11) (Ascenzi *et al*., 2004; Bocedi *et al*., 2004):

$$\mathrm{Log}K_I = \mathrm{Log}K_I^{++} - \mathrm{Log}\{([H^+] + K^*_{lig})/([H^+] + K^*_{unl})\} +$$

$$+ \mathrm{Log}\{([H^+] + K^{**}_{unl})/([H^+] + K^{**}_{lig})\} \quad (11)$$

where K^*_{lig}, K^*_{unl}, K^{**}_{lig} e K^{**}_{unl} are the apparent acidic dissociation constant referring to the acid-base equilibrium of two amino-acid residues (denoted as K^* for the first and as K^{**} for the second residue) for the enzyme-free (K_{unl}) and for the enzyme:inhibitor-bound (K_{lig}) from *L. infantum*, respectively.

MALDI-TOF/TOF analysis. Papain digestion was independently performer on each inhibitor sample by adding approximately 1µM of enzyme and incubating at RT for 1 h. Peptide mixture was micropurified by ZipTip µC18 pipette tips (Millipore). Coelution was performed directly onto a MALDI target with 0.6 µL of CHCA matrix (5 mg/mL in 50% ACN, 0.1% TFA) and allowed to air-dry at room temperature. MS and MS/MS analyses were performed on an Applied Biosystems 4800 MALDI-TOF/TOF.

Data were acquired in positive reflector mode with five spots of standard mixtures (ABI 4700 Calibration Mixture) for calibration. Mass spectra were obtained from each spot by 30 subspectra accumulation (each consisting of 50 laser shots) in the m/z interval ranging between 800 and 4000. For MS/MS spectra, the collision energy was 1 keV and the collision gas was air. The interpretation of both the MS and MS/MS spectra was carried out by using the GPS Explorer software (Version 1.1, Applied Biosystems), which acts as an interface between the Oracle database containing raw spectra and a local copy of the MASCOT search engine (Version 1.8). A monoisotopic mass list from each protein spot was obtained from the MALDI-TOF data according to default parameters. Peptide mass fingerprints were used to search for protein candidates in Swiss-Prot database (Version 54.2) and using the MASCOT software (www.matrixscience.com Version 2.2) according to the following parameters: one missed cleavage permission, cysteine carboamidomethylated and 50ppm measurement tolerance. Oxidation at methionine (variable modification) was also considered. No post-translational modifications were allowed.