Expression and alteration of the S₂ subsite of the *Leishmania major* cathepsin B-like cysteine protease

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The mature form of the cathepsin B-like protease of *Leishmania major* (LmajcatB) is a 243 amino acid protein belonging to the papain family of cysteine proteases and is 54% identical to human-liver cathepsin B. Despite the high identity and structural similarity with cathepsin B, LmajcatB does not readily hydrolyse benzyloxycarbonyl-Arg-Arg-7-amino-4-methyl coumarin (Z-Arg-Arg-AMC), which is cleaved by cathepsin B enzymes. It does, however, hydrolyse Z-Phe-Arg-AMC, a substrate typically cleaved by cathepsin L and B enzymes. Based upon computer generated protein models of LmajcatB and mammalian cathepsin B, it was predicted that this variation in substrate specificity was attributed to Gly²³⁴ at the S₂ subsite of LmajcatB, which forms a larger, more hydrophobic pocket compared with mammalian cathepsin B. To test this hypothesis, recombinant LmajcatB was expressed in the *Pichia pastoris* yeast expression system. The quality of the recombinant enzyme was confirmed by kinetic characterization, N-terminal sequencing, and Western blot

analysis. Alteration of Gly²³⁴ to Glu, which is found at the corresponding site in mammalian cathepsin B, increased recombinant LmajcatB (rLmajcatB) activity toward Z-Arg-Arg-AMC 8-fold over the wild-type recombinant enzyme ($k_{\text{cat}}/K_m = 3740 \pm 413 \text{ M}^{-1} \cdot \text{s}^{-1}$ versus $472 \pm 72.4 \text{ M}^{-1} \cdot \text{s}^{-1}$). The results of inhibition assays of rLmajcatB with an inhibitor of cathepsin L enzymes, K11002 (morpholine urea-Phe-homoPhe-vinylsulphonylphenyl, $k_{\text{inact}}/K_i = 208\,200 \pm 36\,000 \text{ M}^{-1} \cdot \text{s}^{-1}$), and a cathepsin B specific inhibitor, CA074 [*N*-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline, $k_{\text{inact}}/K_i = 199\,200 \pm 32\,900 \text{ M}^{-1} \cdot \text{s}^{-1}$], support the findings that this protozoan protease has the P₂ specificity of cathepsin L-like enzymes while retaining structural homology to mammalian cathepsin B.

Keywords: *Pichia pastoris* expression, protozoan parasite, site-directed mutagenesis

INTRODUCTION

Cysteine proteases play important roles in the development, replication, metabolism, and infectivity of parasitic protozoa [1,2]. Inhibitors of cysteine proteases inhibit the growth and replication of *Plasmodium falciparum* [3,4], *Trypanosoma cruzi* [5,6], *Leishmania mexicana* [7,8], and *Leishmania major* [9] *in vitro*, and have been shown to delay and reduce the extent of disease in mice infected with *Leishmania* [9,10]. Cysteine proteases from parasitic protozoa are therefore considered potential targets for the structure-based approach of drug design for diseases such as malaria, Chagas' disease, and leishmaniasis [11–14]. These parasitic diseases result in the morbidity and mortality of millions of people worldwide, and because no parasite vaccine is available, considerable attention has turned toward the search for new antiparasitic drugs.

The *L. major* cathepsin B (LmajcatB)-like protease is both a target for inhibitor development as well as an interesting example of structure-function variation within the papain family of cysteine proteases. Although structurally homologous to other cathepsin B, it does not readily hydrolyse benzyloxycarbonyl-Arg-Arg-7-amino-4-methyl coumarin (Z-Arg-Arg-AMC) [14], a substrate typically cleaved by cathepsin B [15]. It does, however, hydrolyse Z-Phe-Arg-AMC ($K_m = 7 \mu\text{M}$, [14]), a substrate cleaved by both cathepsin L and B enzymes.

A homology-based structural model of LmajcatB predicts that the difference in substrate specificity to mammalian cathepsin B enzymes lies in the S₂ substrate binding pocket [14]. Unlike rat

and human cathepsin B which have the negatively charged residue Glu²⁴⁵ (mammalian cathepsin B numbering) in the S₂ pocket [15–17], the LmajcatB-like enzyme has Gly²³⁴ at the corresponding position, resulting in a larger, more hydrophobic pocket compared with mammalian cathepsin B [14].

To confirm the predicted structural basis for this unusual substrate specificity and to test the hypothesis that alteration of the Gly²³⁴ is key to this enzyme's preference for a Phe at the P₂ site, we expressed the cathepsin B-like protease of *L. major* in the *Pichia pastoris* yeast expression system. The specificities of the recombinant wild-type and mutant enzyme were compared using the two substrates Z-Arg-Arg-AMC and Z-Phe-Arg-AMC, and with K11002 (morpholine urea-Phe-homoPhe-vinylsulphonyl phenyl), an inhibitor of cathepsin L and CA074 [*N*-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline], an inhibitor of cathepsin B.

MATERIALS AND METHODS

Materials

The synthetic substrates Z-Phe-Arg-AMC and Z-Arg-Arg-AMC were purchased from Enzyme Systems Products (Livermore, CA, U.S.A.). The pseudopeptide vinylsulphone inhibitor K11002 was synthesized and generously provided by Dr. James Palmer (Arris Pharmaceuticals, South San Francisco, CA, U.S.A.). The irreversible inhibitor CA074 was a kind gift from Taisho Pharmaceutical Co. (Saitama, Japan). Mammalian cathepsin B from

Abbreviations used: rLmajcatB, recombinant *Leishmania major* cathepsin B; Z, benzyloxycarbonyl; AMC, 7-amino-4-methyl coumarin.

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bovine spleen was purchased from Sigma Chemicals. Restriction endonucleases and *Pwo* polymerase were from Boehringer Mannheim. The expression vector pPIC9 and GS115 strain of *P. pastoris* were from InVitrogen (San Diego, CA, U.S.A.).

Expression in *P. pastoris*

The LmajcatB was amplified as a proform from genomic DNA using the sense primer 5'-ATA GGA TCC ACC ATG GCG TAC GTA AAG CCG AGT GAC-3' and antisense primer 5'-CGG AAT TCG ACG TCT AGA TGT CTA CTC CTG CGC-3' in PCR. Amplification was carried out in 50 μ l reactions containing 100 ng of genomic DNA, 10 mM Tris/HCl, pH 8.85, 25 mM potassium chloride, 5 mM ammonium sulphate, 2 mM magnesium sulphate, 0.2 mM dNTP, 1.25 units of *Pwo* polymerase, 3% (v/v) DMSO, and 50 pmol of each primer, for 35 cycles of 30 s at 94 °C, 1 min at 60 °C, 1 min at 72 °C. This product was cloned into pBluescriptKS and sequenced. *Sna*BI and *Eco*RI digestion allowed subcloning into the *P. pastoris* yeast expression vector, pPIC9, which forms a fusion of the preproregion of the α -mating factor to the proform of LmajcatB. Procedures for expressing secreted protein in *P. pastoris* were followed as described by the manufacturer, InVitrogen. Briefly, 10 μ g of pPIC9 containing the LmajcatB construct was digested with *Bgl*II and electroporated into GS115 cells and screened for Mut^s (methanol utilization slow) on minimal methanol plates. Integration of the constructs into the yeast genome was confirmed by PCR using 5'AOX1 and 3'AOX1 primers. Transformants were screened for expression in small-scale cultures (10 ml) and analysed by SDS/PAGE and enzyme assays. Cells were grown in 200 ml of buffered minimal glycerol (BMG) medium at pH 6.4 to an attenuance (D_{600}) of approx. 20, pelleted and induced in four 25 ml aliquots of buffered minimal methanol (BMM) medium in 50 ml screw-cap tubes. Methanol was added to a final concentration of 0.5% every 24 h. Under these conditions active enzyme was obtained after 5 to 7 days.

To express active enzyme more efficiently, a second approach was attempted. The second construct was engineered so that the proregion included a KEX2 cleavage site (KR) directly upstream from the N-terminus of recombinant LmajcatB (rLmajcatB) (Figure 1). Residues GVSLE of the α -mating factor replaced the residues LQQD at the C-terminus of the proregion of rLmajcatB, resulting in the sequence GVSLEKR just upstream of the N-terminus of rLmajcatB. Introduction of this sequence between the proregion and the N-terminus of rLmajcatB was accomplished using the oligonucleotides 5'-TCT CTC GAG AAA AGA

CTA CCA GAA TTT TTT GAC GCC GCC-3' and 5'-TCT TTT CTC GAG AGA TAC CCC TTC CTC CAC AG-3' in PCR.

Western blot analysis

Culture media (50 μ g) with and without rLmajcatB, and purified native LmajcatB (2 μ g) were separated by SDS/PAGE [12% (w/v) gel] and electroblotted on to Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.) using 10 mM CAPS, pH 10.0, for 1 h at 100 V. The membrane was blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline, pH 7.5, and incubated in a 1:1000 dilution of polyclonal rabbit antibody raised against native LmajcatB. Specific protein was detected by ECL (Amersham, Arlington Heights, IL, U.S.A.) according to the manufacturer's protocol.

Site-directed mutagenesis

Gly²³⁴ was mutated to Glu using the oligonucleotides 5'-GAG AGT GAG GGC GTT GCT GGT ATA C-3' and 5'-GTA TAC CAG CAA CGC CCT CAC TCT CA-3', which introduced a *Bst*1107I site to facilitate screening. All mutations were made by recombinant PCR [18] using *Pwo* polymerase and the original proform of rLmajcatB in pBluescript as a template. A consensus *N*-glycosylation site in the catalytic portion of the enzyme, Asn-Thr-Thr, was removed by mutating Thr¹²⁴ to Ala using the oligonucleotides 5'-TGC AAT ACC GCC TGT GAG AGA AGC-3' and 5'-CTC ACA GGC GGT ATT GCA TTT AGG-3'.

N-terminal sequence

Recombinant protein samples were separated by SDS/PAGE [12% (w/v) gels] and electroblotted on to Immobilon-P membrane following the manufacturer's procedures. Samples were then submitted to the Protein and Nucleic Acid Facility, Beckman Center (Stanford University, Palo Alto, CA, U.S.A.) for N-terminal sequencing by Edman degradation.

Enzyme and inhibition assays

Kinetic and inhibition constants were determined in a reaction buffer containing 100 mM sodium acetate, pH 5.5, 10 mM dithiothreitol, 0.1% Triton X-100, 1 mM EDTA, and 0.5% DMSO. Proteolytic activity was monitored by the hydrolysis of 7-amino-4-methyl coumarin from either of the synthetic peptide substrates Z-Phe-Arg-AMC or Z-Arg-Arg-AMC. Initial rates were measured in microtiter plates using a Labsystem Fluoroscanner II spectrofluorometer at 460 nm emission and an excitation wavelength of 355 nm. Enzyme concentration was determined by active-site titration using E-64c, an epoxysuccinyl peptide [19]. Data were fitted using the Ultrafit non-linear regression analysis software (Biosoft, Ferguson, MO, U.S.A.). All assays were performed at 25 °C.

Wild-type and Gly²³⁴ to Glu (G234E) mutant enzymes were compared by measuring the enzyme activity at different pHs in 10 mM dithiothreitol, 0.1% Triton X-100, 1 mM EDTA, 0.5% DMSO, and 100 mM of the following buffers: sodium acetate (pH 4.5, 5.0 and 5.5), Mes (pH 6.0 and 6.5), Pipes (pH 7.0), and Tris (pH 7.5, 8.0 and 8.5). Enzyme concentrations were 2 nM when assaying with 50 μ M Z-Phe-Arg-AMC. In order to measure activity with Z-Arg-Arg-AMC as substrate, both enzyme and substrate concentrations were increased 5-fold to 10 nM and 250 μ M, respectively.

For K_m and k_{cat} determinations, wild-type rLmajcatB enzyme concentrations were 2.3 nM and 18.6 nM for the substrates Z-Phe-Arg-AMC and Z-Arg-Arg-AMC respectively. In the G234E

Figure 1 The two constructs engineered to express the LmajcatB enzyme in *P. pastoris*

Construct 1 was generated by following protocols described by InVitrogen and is a fusion of the preproregion of the α -mating factor (α MF) to the proform of rLmajcatB. Construct 2 was engineered to include a second KEX2 cleavage site directly upstream from the N-terminus. The residues LQQD were deleted and replaced with GVSLEKR of the α MF.

mutant, enzyme concentrations were 3.6 nM and 14.5 nM for the two substrates respectively.

Enzyme assays using irreversible inhibitors were performed under pseudo-first-order conditions to monitor time-dependent inactivation as described previously [9]. For each assay, six different inhibitor concentrations were used in duplicate in at least three independent experiments. In the case of wild-type rLmajcatB, the enzyme concentration was 2.3 nM and the substrate concentration was 10 μ M Z-Phe-Arg-AMC. For the G234E mutant, the enzyme concentration was 3.6 nM and the substrate concentration was 7.5 μ M Z-Phe-Arg-AMC.

RESULTS

Expression of the rLmajcatB-like protease

Two constructs were engineered to express the *L. major* enzyme (Figure 1). The first construct resulted in three forms of rLmajcatB which were detected in growth media after 7 days of methanol induction (Figure 2A). N-terminal amino acid sequence analysis revealed that the proform (beginning at residue 29p) of the enzyme (molecular mass of 40 kDa) contained residues consistent with those expected after cleavage by KEX2, and the gene product of *Ste13* of *P. pastoris* as engineered by InVitrogen (first arrow, Figure 3). The intermediate form contained a proregion that was internally cleaved resulting in a protein with a molecular mass of 34 kDa (second arrow, Figure 3). The recombinant enzyme with a molecular mass of 31 kDa had an N-terminal extension of five residues relative to the mature form of the enzyme (third arrow, Figure 3). This is analogous to the six-residue extensions observed when rat procathepsin B expressed in *S. cerevisiae* is activated by cathepsin B [20]. The relatively large shift between the middle and lower bands in Figure 2(A, lanes 2 and 5), may be due to possible N-linked glycosylation at position 89 of the intermediate form.

All three forms of rLmajcatB were observed in the media even after 9 days of induction. In an effort to produce recombinant enzyme that did not contain additional residues left by *Pichia* processing, a second construct was engineered to include a KEX2

Figure 2 Expression of rLmajcatB in *P. pastoris* in (A) construct 1 and (B) construct 2

(A) Left panel, Coomassie Blue stained SDS/polyacrylamide gel. Right panel, Western blot using rabbit antiserum to purified native LmajcatB. Lanes 1 and 4, pPic9 culture media; lanes 2 and 5, culture media containing the 3 forms of recombinant LmajcatB following 7 days of induction; lanes 3 and 6, purified native LmajcatB. Protein standards are the BioRad Low Molecular Weight Protein Standards (kDa) (B) Left panel, Coomassie Blue stained SDS/polyacrylamide gel with recombinant protein expressed after 3 days of induction (Lane 1). Right panel, Western blot using the same antibody as in Figure 2(A). Lane 2, pPic9 culture media; Lane 3, culture media with the KEX2 construct.

Figure 3 Amino acid alignment of the *L. major* cathepsin B-like enzyme (LmajcatB) with rat cathepsin B (catb-rat) and human cathepsin B (catb-hum)

Arrows indicate the N-termini of the three secreted forms of recombinant LmajcatB that were processed after 7 days of induction. The letter p after the residues indicates the pre- and proregion of LmajcatB. Bold-faced letters indicate residues that were altered by site-directed mutagenesis. Asterisks indicate the three residues of the catalytic triad; vertical lines indicate identity and double dots denote conserved amino acid changes.

cleavage site upstream from the N-terminus. Residues GVSLE of the α -mating factor and the KEX2 cleavage site (KR) replaced the last four residues (LQQD) of the rLmajcatB proregion (Figure 1). The rationale was that by placing the residues KR just at the start of the N-terminus of the mature portion of rLmajcatB, a fully processed form of enzyme would be produced.

On Day 1 of culture growth, the only secreted form of the recombinant protein was the proform of the enzyme. On Day 3 following induction, the proform was processed to the active form of the enzyme which was the only form present (Figure 2B). N-terminal sequence information of this protein revealed that KEX2 had not processed the enzyme at the expected cleavage site. Instead, the active recombinant enzyme had an eight residue extension (EGVSLEKR) at its N-terminus which may have been processed by a yeast enzyme or by autoprocessing. While the introduction of a second putative KEX2 site did not result in cleavage at this specific site, it did yield a form of the proenzyme which was processed more effectively. This result indicates that manipulation of the region linking the propeptide to the mature enzyme can modulate processing.

Since this KEX2 construct produced a single active product, this clone was used to express recombinant protease for the enzyme assays and in the site-directed mutagenesis study. The yield of this secreted rLmajcatB, as determined by active-site titration with E-64, was 33 mg per litre of culture after 3 days of induction. Maximal activity of the second construct was observed after 3 days of induction, which was in marked contrast to the first construct which yielded similar activity after 9 days of induction. Use of the second KEX2 construct has not been tried

Figure 4 Enzyme activity of wild-type rLmajcatB (■) and G234E mutant rLmajcatB (▲) assayed at various pHs with the two substrates, Z-Phe-Arg-AMC (solid lines) and Z-Arg-Arg-AMC (broken lines)

Enzyme and substrate concentrations with Z-Phe-Arg-AMC were 2 nM and 50 μ M, respectively, and 10 nM and 250 μ M, respectively with Z-Arg-Arg-AMC. FI U/min, fluorescent units per min.

with other cysteine proteases, but it may prove to be useful if expression results in multiple forms of the recombinant enzyme.

Wild-type and mutant enzyme

Wild-type rLmajcatB and G234E mutant enzyme were assayed with the two substrates, Z-Phe-Arg-AMC and Z-Arg-Arg-AMC. Wild-type rLmajcatB had a K_m of 10.2 μ M with Z-Phe-Arg-AMC at pH 5.5, which was similar to that for the native enzyme ($K_m = 7.0 \mu$ M). Over the entire pH range, wild-type and mutant enzyme displayed similar activities toward Z-Phe-Arg-AMC, with optimal activity at pH 5.5 (Figure 4). However, the wild-

type enzyme did not readily hydrolyse Z-Arg-Arg-AMC, which was consistent with results using the native enzyme [14].

The k_{cat}/K_m value for the wild-type enzyme was approx. 4-fold higher with Z-Phe-Arg-AMC as substrate compared with the mutant enzyme, but was 8-fold lower than the value for the mutant enzyme with Z-Arg-Arg-AMC (Table 1).

Inhibition of wild-type and mutant enzyme

Inhibition of wild-type rLmajcatB by K11002 [21] and CA074 [22] revealed that both were excellent inhibitors (Table 1). The k_{inact}/K_i value for wild-type rLmajcatB with K11002 was approx. 150-fold higher than it was for mammalian cathepsin B. Inhibition of mammalian cathepsin B by CA074 ($k_{inact}/K_i = 133\,330 \pm 14\,000 \text{ M}^{-1} \cdot \text{s}^{-1}$) was similar to that for wild-type rLmajcatB ($k_{inact}/K_i = 199\,200 \pm 32\,900 \text{ M}^{-1} \cdot \text{s}^{-1}$). The large difference in k_{inact}/K_i values for wild-type rLmajcatB and mammalian cathepsin B with K11002 may reflect the inhibitor's specificity for the S_2 pocket. The similar k_{inact}/K_i values for wild-type rLmajcatB and mammalian cathepsin B with CA074, on the other hand, may reflect the inhibitor's specificity for the two histidine residues in the occluding loop of cathepsin B-like enzymes.

DISCUSSION

The LmajcatB enzyme is one of the most primitive known members of this subfamily, yet it has high sequence similarity with other cathepsin B proteases, including mammalian cathepsin B [23]. Molecular structures based upon homology modelling suggest that the LmajcatB enzyme has the 'occluding loop' containing the two histidine residues (H101 and H102) which are responsible for the exopeptidase activity and an interdomain interface similar to other cathepsin B enzymes [14,16,17,24]. Despite the high identity and structural similarity with other cathepsin B enzymes, this protozoan protease has a very different substrate specificity.

Unlike the mammalian cathepsin B enzymes which have the ability to hydrolyse substrates with Phe-Arg and Arg-Arg in the P_2 - P_1 position, LmajcatB does not readily hydrolyse Z-Arg-Arg-AMC. This preference for Phe in the P_2 position, which is more typical of cathepsin L enzymes, is attributed to the glycine at residue 234 of the protozoan enzyme which corresponds to Ser²⁰⁵ of papain and Glu²⁴⁵ of rat and human cathepsin B (SWISS-PROT accession numbers P00787 and P07858 respectively). This

Table 1 Kinetic data of wild-type rLmajcatB and G234E mutant enzyme with the substrates Z-Phe-Arg-AMC and Z-Arg-Arg-AMC

Inhibition constants were determined using Z-Phe-Arg-AMC as the substrate. *Data from [9].

Substrates ...	Z-Phe-Arg-AMC			Z-Arg-Arg-AMC		
	k_{cat} (s^{-1})	K_m (μ M)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μ M)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Wild-type rLmajcatB	7.6 ± 0.63	10.2 ± 1.44	$746\,100 \pm 121\,900$	0.145 ± 0.020	308 ± 20.0	472 ± 72.4
G234E mutant	6.6 ± 0.99	33.2 ± 9.57	$199\,300 \pm 64\,800$	1.05 ± 0.050	281 ± 28.3	3740 ± 413.0
Inhibitors ...	K11002			CA074		
	k_{inact} (s^{-1})	K_i (μ M)	k_{inact}/K_i ($M^{-1} \cdot s^{-1}$)	k_{inact} (s^{-1})	K_i (μ M)	k_{inact}/K_i ($M^{-1} \cdot s^{-1}$)
Wild-type rLmajcatB	0.0094 ± 0.0009	0.045 ± 0.0069	$208\,200 \pm 36\,800$	0.0069 ± 0.0007	0.035 ± 0.0046	$199\,200 \pm 32\,900$
G234E mutant	0.0100 ± 0.0011	0.186 ± 0.0048	$54\,760 \pm 5850$	0.0068 ± 0.0010	0.022 ± 0.0051	$301\,300 \pm 82\,400$
Cathepsin B*	0.014 ± 0.001	9.8 ± 2.3	$1\,400 \pm 250$	0.020 ± 0.009	0.15 ± 0.06	$133\,330 \pm 14\,000$

difference results in a much larger, hydrophobic S_2 subsite in the LmajcatB enzyme compared with mammalian cathepsin B [14].

By exchanging Gly²³⁴ for Glu in LmajcatB, we showed that protease activity toward a substrate with Arg at the P_2 site, can be increased by as much as 8 times compared with the wild-type enzyme. This single mutation at the S_2 subsite did not affect the enzyme's ability to hydrolyse the substrate typically cleaved by cathepsin L enzymes (Z-Phe-Arg-AMC), but it did enhance the enzyme's ability to hydrolyse a substrate it otherwise does not readily cleave (Z-Arg-Arg-AMC). Thus, alteration of a single residue allowed the protozoan enzyme to display a cathepsin B-like specificity. Interestingly, when the plant protease papain, which also exhibits a preference for Phe over Arg at the P_2 position (904-fold versus 3.6-fold compared with cathepsin B), was mutagenized at the S_2 subsite (Val¹³³ → Ala/Ser²⁰⁵ → Glu), it exhibited cathepsin B-like specificity [25].

Consistent with predictions of the homology-based molecular model, rLmajcatB was inhibited by both an inhibitor of cathepsin L enzymes and a specific cathepsin B inhibitor. The k_{inact}/K_i value for rLmajcatB with K11002, an inhibitor of cathepsin L-like proteases, was comparable to the k_{inact}/K_i values for papain and cruzain, the cathepsin L-like enzyme of the related kinetoplastid parasite, *T. cruzi* [9]. This result is in contrast to the k_{inact}/K_i value for mammalian cathepsin B.

CA074, a derivative of E-64, an epoxysuccinyl peptide, is a specific inhibitor of cathepsin B [22]. CA074 proved to be as effective an inhibitor for the rLmajcatB enzyme as it was for bovine spleen cathepsin B with k_{inact}/K_i values of $199\,200 \pm 32\,900 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $133\,330 \pm 14\,000 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. Thus, the rLmajcatB enzyme has a P_2 specificity similar to that of cathepsin L-like enzymes, yet has structural homology to cathepsin B enzymes, including the occluding loop that is targeted by CA074 [26–29].

This information will aid in the design of specific inhibitors for *Leishmania* cysteine proteases, and serves to validate the use of homology-based modelling of cysteine proteases for initial predictions of active-site specificity and inhibitor design.

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