Cloning, expression and characterisation of an HtrA-like serine protease produced in vivo by *Mycobacterium leprae*

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Members of the high temperature requirement A (HtrA) family of chaperone proteases have been shown to play a role in bacterial pathogenesis. In a recent report, we demonstrated that the gene ML0176, which codes for a predicted HtrA-like protease, a gene conserved in other species of mycobacteria, is transcribed by Mycobacterium leprae *in human leprosy lesions. In the present study, the recombinant ML0176 protein was produced and its enzymatic properties investigated.* M. leprae *recombinant ML0176 was able to hydrolyse a variety of synthetic and natural peptides. Similar to other HtrA proteins, this enzyme displayed maximum proteolytic activity at temperatures above 40°C and was completely inactivated by aprotinin, a protease inhibitor with high selectivity for serine proteases. Finally, analysis of* M. leprae *ML0176 specificity suggested a broader cleavage preference than that of previously described HtrAs homologues. In summary, we have identified an HtrA-like protease in* M. leprae *that may constitute a potential new target for the development of novel prophylactic and/or therapeutic strategies against mycobacterial infections.*

Key words: *Mycobacterium leprae* - HtrA2 - protease - enzymatic activity - FRET peptides - pathogenesis

Infections caused by species of the genus *Mycobacterium* continue to adversely affect the lives of millions worldwide. Leprosy, caused by *Mycobacterium leprae*, remains a public health problem in several developing countries, including Brazil, and is responsible for the legacy of countless numbers of people with permanent physical deformities (WHO 2009b). In addition, tuberculosis, caused by *Mycobacterium tuberculosis*, is responsible for 2-3 million annual deaths (WHO 2009a). Furthermore, infections in immunocompromised individuals caused by opportunistic mycobacterial species, such as *Mycobacterium avium* and *Mycobacterium intracellulare*, belonging to the *M. avium* complex, have gained additional epidemiological importance since the emergence of AIDS (Garcia Garcia et al. 2005). Deciphering the mechanisms implicated in mycobacterial pathogenesis is currently a major challenge in leprosy and tuberculosis research and may eventually lead to the development of new prophylactic and/or therapeutic strategies.

Members of the high temperature requirement A (HtrA) family are envelope-associated serine proteases that perform crucial functions, involving protein quality control in the periplasmic space, acting as both molecular chaperones and proteases (Pallen & Wren 1997, Page & Di Cera 2008). It has been shown in a wide range of bacterial species that HtrA proteases are essential for virulence and survival under environmental stress. In fact, mutations in the *htr*A gene have been shown to affect bacterial tolerance to both thermal and environmental stress and to produce a loss of virulence, as shown in *Porphyromonas gingivalis*, *Chlamydia trachomatis*, *Salmonella typhimurium*, *Streptococcus pyogenes*, *Listeria monocytogenes* and *Burkhoderia cenocepacia* (Jones et al. 2001, Biwas & Biwas 2005, Stack et al. 2005, Mo et al. 2006, Flannagan et al. 2007, Huston et al. 2008, Yuan et al. 2008, Lewis et al. 2009). Biochemical analysis of the three HtrA proteins found in *Escherichia coli*, DegP, DegQ and DegS, has provided insights into their function and regulation (Lipinska et al. 1989, 1990, Kolmar et al. 1996, Krojer et al. 2008). However, the function of this protein family in Gram positive bacteria remains unclear.

A recent comparison of the protease-coding genes present in the genomes of *M. leprae*, *M. tuberculosis*, *Mycobacterium bovis and Mycobacterium paratuberculosis* revealed that three well-conserved putative HtrA-like genes are shared by all four species (Ribeiro-Guimarães & Pessolani 2007). The *htrA2* gene in mycobacterium is part of an operon in which the genes coding for the two-component regulatory system MprA-MprB are located upstream of *htrA2* (Zahrt et al. 2003). Recent studies have shown that *M. tuberculosis* HtrA2 is positively regulated by MprAB (He & Zahrt 2005), which also regulates other stress-response genes (He et al. 2006). Moreover, it was shown that MprAB is required by *M. tuberculosis* for growth *in vivo* (Zahrt & Deretic 2001), suggesting that HtrA members are essential for mycobacterial persistence in the host. This idea is reinforced by recent findings indicating transcription of

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putative *htrA2 (*ML0176) and *htrA4* (ML2659) genes by *M. leprae* isolated from skin biopsies of multibacillary leprosy patients (Ribeiro-Guimarães et al. 2007), as well as expression of the HtrA4 protein by armadillo-derived *M. leprae* (Marques et al. 2008).

Deciphering the biology of *M. leprae* has constituted one of the greatest challenges for microbiologist over time due to its inability to grow in vitro and the absence of experimental models that mimic the disease observed in humans. However, the *M. leprae* genome sequence has recently become available, providing an opportunity to study the enzymes of this microorganism, which are inaccessible for direct study. For the first time, the present study demonstrates that the recombinant *M. leprae* HtrA2 exhibits proteolytic activity toward synthetic and naturally-occurring peptides, revealing physicochemical properties of a typical HtrA-like protease.

MATERIALS AND METHODS

Materials - The fluorescence resonance energy transfer (FRET) peptides used in the present paper (Table I) were synthesised and purified, as described elsewhere (Hirata et al. 1995). The purity and the molecular masses of these peptides were determined by high performance liquid chromatography (HPLC) and by matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MS) (TofSpec-E, Micromass, UK), respectively. Nα-RR-methylcoumarin (MCA), dynorphin A, neurotensin 1-13, bradykinin and angiotensin I peptides as well as the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and aprotinin were purchased from Sigma Co (St. Luis, MO). Trypsin was purchased from Roche Diagnostics (Indianapolis, IN).

Cloning, expression and purification of rHtrA2 - The primers for the *htrA2* gene were constructed to correspond to the 5' and 3' ends of the open reading frame previously identified. The sequences of 5' and 3' primers were 5`-GTTGGAATTCATGATTCCGCCCGGT-3` and 5'-ACAACTCGAGCTCTCGATTAT TAAT-3', respectively. The underlined sequences represent the sites for the restriction enzymes *EcoRI* and *XhoI*, respectively, incorporated into the primers for subsequent cloning. The gene was PCR amplified from *M. leprae* genomic DNA with Deep Vent DNA polymerase. PCR was carried out using a PTC-100™ (MJ Research, Inc) at an annealing temperature of 58°C. The PCR product was gel purified and digested with *EcoRI* and *XhoI* followed by cloning into pET32a-c (+) vector (Novagen, San Diego, CA) previously digested with the same enzymes. Overexpression and purification of the *M. leprae* HtrA2 protein were performed according to the QIAexpressionist manual (QIAGEN, Valencia, CA). Briefly, *E. coli* BL21-AI™ harbouring the recombinant plasmid was grown up to an OD_{600} of 0.8 in ampicillin-containing Luria-Bertani (LB) medium. Expression of rHtrA2 was then induced by addition of 1 mM isopropyl beta-D-thiogalactoside (IPTG) and cultures were incubated at 37°C for another 3 h. Cells were harvested, ressuspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris·Cl pH 8.0) and disrupted by sonication. Debris was removed by centrifugation at

TABLE I

Kinetic parameters for the degradation of substrates derived from Abz-GFSPFRQ-EDDnp by rHtrA2

	Substrates (Abz-…-EDDnp)								K_m [b] (μM)	k_{cat} (s^{-1})	k_{cat}/K_m $(s \cdot mM)^{-1}$
N	P_6	P_5	P_4	P_3	P_2	P_1	↓[a]	P'_1			
1	G	F	S	P	R	R	↓	Q	3.16 ± 0.43	63.00 ± 3.0	19.90
2	G	F	S	P	D	R		Q	nh	nh	nh
3	G	F	S	P	F	R	↓	Q	6.96 ± 0.64	4.80 ± 0.30	0.68
4	G	F	S	P	A	R	↓	Q	3.40 ± 0.36	9.40 ± 0.50	2.76
5	G	F	S	P	S	R	↓	Q	9.48 ± 0.90	27.00 ± 3.00	2.85
6	G	F	S	P	P	R	↓	Q	4.58 ± 0.90	4.20 ± 0.30	0.92
7	G	F	S	I	F	R	↓	Q	2.09 ± 0.20	19.00 ± 1.00	9.13
8	G	F	S	F	F	R	↓	Q	7.78 ± 0.78	26.00 ± 2.00	3.34
9	G	F	S	L	F	R	↓	Q	2.04 ± 0.19	6.60 ± 0.50	3.23
10	G	F	P	P	F	R	↓	Q	5.30 ± 0.41	3.70 ± 0.25	0.70
11	G	F	F	P	F	R	↓	Q	1.96 ± 0.20	10.00 ± 1.00	5.10
12	G	F	R	P	F	R	↓	Q	3.18 ± 0.30	31.00 ± 3.00	9.75
13	G	F	S	P	F	R	↓	Q	6.96 ± 0.64	4.80 ± 0.30	0.69
14	G	F	A	P	F	R	↓	Q	6.27 ± 0.60	5.20 ± 0.40	0.83
15	G	F	E	P	F	R	↓	Q	2.83 ± 0.28	2.00 ± 0.15	0.71

a: cleavage sites determined by mass spectrometry analysis are indicated by ↓; *b*: assays were carried out in a 50mM phosphate buffer, pH 8.0, at 37°C. The kinetic parameters were obtained in the presence of 1/10-10 times the K_m value of peptide substrate and 10-100 nM of *rHtrA2*, with a substrate consumption of less than 5%. The velocity was recorded for 5-15 min and the parameters were calculated as mean value (± SD). All enzymatic assays were performed in triplicate. nh: no hydrolysis detected.

10,000 *g* for 15 min at 4ºC and rHtrA2 was purified from the supernatant by adding 1 mL of a 50% Ni-NTA slurry (QIAGEN) pre-equilibrated in lysis buffer to 4 mL of the lysate. After incubation for 1 h at 4ºC, the lysate-Ni-NTA mixture was loaded into a column. Unbound proteins were removed by washing the column with two column volumes of lysis buffer. The column was washed twice with 4 mL of 100 mM NaH_2PO_4 and 10 mM Tris-Cl, pH 6.3 and the recombinant protein was eluted with four column volumes of 100 mM NaH_2PO_4, pH 4.5. Fractions were analysed by SDS-PAGE and Western Blot. Nitrocellulose membranes were probed with an anti-6xHis tag antibody (New England Biolabs, Ipswich, MA). The purified enzyme was stored in 50% glycerol at -20°C.

HPLC analysis of rHtrA2 - The fractions eluted from the Ni-NTA matrix enriched in rHtrA2 were concentrated at a flow rate of 1.0 mL/min in a Superdex™ 75 gel filtration column (GE Healthcare Life Science do Brasil, São Paulo, Brazil) pre-equilibrated in 0.05 M sodium phosphate and 0.15 M NaCl, pH 6.8. Protein-containing fractions were analysed by SDS-PAGE followed by silver staining. Protein concentration was determined according to Bradford (Bradford 1976) using bovine serum albumin as a standard.

MS analysis of recombinant HtrA2 - The purified recombinant HtrA2 protein was digested with trypsin. The resulting peptides were then applied to a 0.2 x 50 mm C_{18} capillary reverse phase column (Michrom BioResources, Auburn, CA) and eluted with an increasing acetonitrile gradient using a MicroPro capillary HPLC system (Eldex Laboratories, Napa, CA). The reverse-phase eluent was introduced directly into a Finnigan LCQ electrospray MS (Thermoquest, San Jose, CA). The peptides were analysed by MS or MS/MS followed by a search in the *M. leprae* database with Xcalibur Bioworks 3.1 turbo SEQUEST software (ThermoFinnigan, San Jose, CA) under the conditions and parameters previously described (Biet et al. 2007).

Enzyme assays of recombinant HtrA2 - The hydrolyses of the fluorescent substrates (stock solution in 10% DMSO) were conducted at 37°C in 50 mM Tris-HCl, pH 8.0, containing 20 mM NaCl. Hydrolysis of Nα-RR-MCA and the FRET substrates were monitored by measuring fluorescence at λem = 460 nm and λex = 380 nm and λem = 420 nm and λex = 320 nm, respectively, with a Hitachi F-2000 spectrofluorimeter, as previously described (Oliveira et al. 2001). The slope was converted into moles of hydrolysed substrate per minute, based on the fluorescence curves of standard peptide solutions before and after total enzymatic hydrolysis with trypsin. A 1-cm path length cuvette containing 1 mL of substrate solution was placed in a thermostatically controlled cell compartment prior to adding the enzyme solution. Enzyme concentrations ranged from 10-100 nM and FRET substrates from 1/10-10 times the K_m value. The increase of fluorescence over time was continuously recorded for 5-15 min. The kinetic parameters were calculated according to Wilkinson (Wilkinson 1961). One unit of recombinant HtrA2 activity was defined as the amount of enzyme needed to hydrolyse 1 μmoL of Nα-RR-MCA in 1 min. As previously described, the inner-filter effect of the FRET substrates was corrected by an empirical equation (Oliveira et al. 2001).

HPLC analysis of peptides hydrolysed by rHtrA2 - Peptide solutions (50 μM) in 50 mM Tris-HCl, pH 8.0, containing 20 mM NaCl were incubated with rHtrA2 (10 nM) at 37°C for 4 h. Samples (100 μL) were periodically withdrawn for HPLC analysis. The hydrolysis products were fractionated by reverse-phase HPLC (Class VP, Shimadzu), manually collected and submitted to MS analysis. The HPLC conditions used for the analytical procedure included 0.1% trifluoroacetic acid in water (solvent A) and acetonitrile-solvent A (9:1) as solvent B. The separations were performed at a flow rate of 1 mL/min using a JT Baker C_{18} column (4.6 x 300 mm) and a 10-80% gradient of B for 30 min. Fractions were monitored for the presence of peptides fragments using an SPD-10AV Shimadzu uv/vis detector (214 nm) and a RF-10Ax fluorescence detector (λ_{em} = 420 nm and λ_{ex} = 320 nm).

MS analysis of peptide substrates hydrolysed by rHtrA2 - Peptide fragments were detected by scanning from a m/z of 50-2.000 at 6 s/scan with a 31 V cone. Product-ions from the MS/MS experiments were detected during several scans through the appropriated mass range for each situation using high energy (25 eV) for single-charged precursor ions and low-collision energy (15 eV) for multiple-charged precursor ions. No tandem MS was recorded for peptides smaller than four amino acid residues. Scissile bonds were deduced from the amino acid compositions of the fragments.

Effect of pH, temperature and inhibitors on peptidase activity - The pH studies were performed in 50 mM sodium citrate buffer (pH 3.0 - 5.3), 50 mM sodium phosphate buffer (pH 5.2 - 7.5) and 50 mM Tris-HCl buffer (pH 7.3 - 10) containing 20 mM NaCl. The stock solutions and the working concentration of the synthetic inhibitors used in the characterisation of HtrA2 proteolytic activity matched those previously described (Dunn et al. 1989). The pH, temperature and peptidase inhibition effects were determined by fluorometric assay using the peptide Nα-RR-MCA (10 μM) as a substrate. The temperature range used was 28-60°C and the enzymatic reactions were performed in 50 mM phosphate buffer, pH 8.0.

RESULTS AND DISCUSSION

Cloning, expression and purification of M. leprae HtrA2 - The gene coding for a putative HtrA2 protease was amplified from *M. leprae* genomic DNA and successfully cloned into the pET32 vector. *E. coli* BL21 harbouring the recombinant vector was grown in LB medium containing ampicillin and induced by IPTG. The soluble hexa-histidine-tagged fusion protein was purified in one step by immobilised, metal-affinity chromatography from cell lysates using the Ni-NTA resin. A single band corresponding to the recombinant HtrA2 protein with the expected MW of 52.3 kDa was observed in a silver stained SDS-PAGE gel (Fig. 1A). The purified protein was transferred to a nitrocellulose

membrane and recognised by the anti-6xHis tag antibody (Fig. 1A). A high degree of purity of the recombinant ML0176 was ensured by loading the protein onto a Superdex 75 gel filtration column. The elution profile (Fig. 1B) revealed a single homogeneous peak corresponding to the enzyme, as confirmed by SDS-PAGE followed by trypsin digestion and MS/MS analysis. The following peptides, all matching the predicted amino acid sequence of *M. leprae* ML0176 were sequenced: ^{78}KVVPSVVMLETDLGRQ^{91}, ^{132}KTTVTFFDGRT^{140}, ^{141}RTASFTVVGADPTSDIAVVRV^{159}, ^{160}RVQSISGLPPITMGSSADLLRV^{178}, ^{339}RLISSADALVAAVRS^{351} and ^{359}KVSLTYQDQSGSSRT^{371}.

Primary sequence analysis of M. leprae rHtrA2 - Members of the HtrA family share a conserved trypsin-like protease domain and at least one C-terminal PDZ domain, which mediates specific protein-protein interactions and is involved in substrate recognition (Pallen & Wren 1997, Clausen et al. 2002, Kim & Kim 2005). Fig. 2 shows the amino-acid sequence alignment among *M. leprae* HtrA2, HtrA2 from *M. tuberculosis* H37Rv and DegS from *E. coli*. *M. leprae* HtrA2 shares 70% identity with the *M. tuberculosis* H37Rv HtrA2 and 26% identity with DegS. This alignment also indicates conservation in *M. leprae* HtrA2 of common features of the HtrA family members, such as a signal peptide, a trypsin-like domain with the conservation of a putative catalytic triad consisting of the amino acid residues His182, Asp224 and Ser305 and a consensus PDZ domain at the C-terminal region.

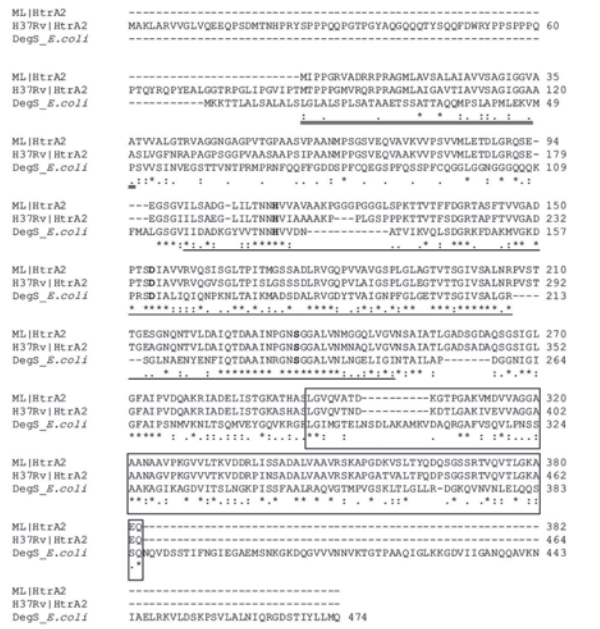

Fig. 2: alignment of the amino acid residues from *Mycobacterium leprae* and *Mycobacterium tuberculosis* HtrA2 *and Escherichia coli* DegS. The signal sequence predicted (CBS 2007) for HtrA2 of *M. leprae* is double underlined (residues 1-36) and the domain for the trypsin family (IPR001254) is underlined (residues 102-249). The conserved residues that likely comprise the catalytic triad are in bold. The PDZ domain (IPR001478) is indicated by an open box (residues 287-382).

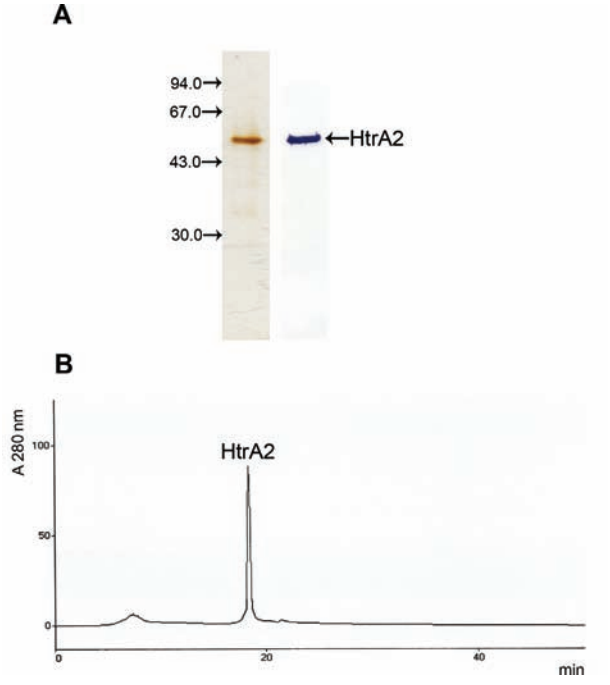

Fig. 1: purification of *Mycobacterium leprae* high temperature requirement A (HtrA)2. A: SDS-PAGE and Western blot showing the rHtrA2 (4 µg) obtained after purification by immobilised metal-affinity chromatography on Ni-NTA resin. The gel was stained with silver and Western blot was developed with anti-His tag antibody. The positions of molecular size markers are shown on the left; B: elution profile of the purified rHtrA protein from a Superdex G75 gel filtration column.

Analysis of cleavage specificity and kinetic parameters - HtrA proteins belong to the serine protease clan PA composed of serine proteases that display the following three main types of activity: trypsin-like (the cleavage of the amide bond occurs after Arg or Lys at P1), chymotrypsin-like (cleavage occurs following one of the hydrophobic amino acids at P1) and elastase-like (cleavage following an Ala at P1). A variety of substrates, including synthetic and natural peptides, were then tested for cleavage by the recombinant ML0176 protein.

A collection of 15 synthetic FRET peptides of seven amino acid residues derived from the bradykinin sequence was used as substrates. These peptides allow convenient determination of kinetic parameters because hydrolysis of any peptide bond of the FRET substrates can be easily monitored (Hirata et al. 1995). Table I displays the K_m, k_{cat} *and* k_{cat}/K_m values that were determined for the hydrolysis of the FRET substrates by *M. leprae* rHtrA2. All cleaved peptides were hydrolysed at the R-Q bond. The catalytic efficiency (k_{cat}/K_m) varied from no hydrolysis of the FRET-2 peptide to 19.90 $(s \cdot mM)^{-1}$ of the FRET-1 peptide. Among the FRET-1 to 6 peptides with variable amino acid residues at the P2 position, the best substrate was FRET- 1, showing an Arg residue in this position. In contrast, the presence of Asp at position P2 (FRET-

2) resulted in a total absence of interaction between the enzyme and the substrate. The FRET-7 to 9 peptides revealed that rHtrA from *M. leprae* preferred amino acid residues with an aliphatic chain, like Ile or Leu, instead of Phe at P3. Among the FRET-10 to 15 peptides with amino acid residue changes at P4, the best substrate was FRET-12 with an Arg residue at this position.

Five naturally-occurring peptides of various sizes and amino acid sequences were also tested for cleavage by the enzyme. Hydrolysis was monitored by HPLC and the results are summarised in Table II. The most susceptible substrate was neurotensin, which presented one minor (R8↓R9) and two major cleavage sites (Y3↓E4 and Y11↓I12). When dynorphin was the substrate, rHtrA2 showed a preference for hydrolysis involving basic amino acid residues in a P1 position (R6↓R7 and R7↓I8). *M. leprae* rHtrA2 failed to hydrolyse oxytocin, angiotensin I and bradykinin, although the sequences of the latter two peptides contain basic residues.

The proteolytic activity profile displayed by *M. leprae* rHtrA2 over the FRET and naturally occurring peptides suggest that this enzyme recognises the substrate via P1-S1 and through an extended interaction site. In contrast, other HtrA family member activity has been predicted to depend solely on what is present in the P1 position (Kim & Kim 2005, Hauske et al. 2009). Moreover, *M. leprae* rHtrA2 was able to cleave after basic and hydrophobic residues. In this way, *M. leprae* rHtrA2 seems to display a broader cleavage specificity than has been described in the context of *E. coli* DegP and DegS, which have shown restricted cleavage preference after hydrophobic residues (Clausen et al. 2002, Jones et al. 2002).

Physicochemical properties - Further characterisation of *M. leprae* rHtrA2 was performed using Nα-RR-MCA, which has only one cleavage site at the R-MCA position, as a substrate. Fig. 3A shows that Nα-RR-MCA was efficiently cleaved by *M. leprae* rHtrA2.

To confirm the class of the protease, tests were performed to determine whether the activity of *M. leprae* rHtrA2 was affected by PMSF and aprotinin, two classical inhibitors of serine peptidases. The proteolytic activity of *M. leprae* rHtrA2 was markedly reduced by both inhibitors and at 4.2 mU aprotinin was able to completely inactivate the enzyme (Fig. 3A).

The effect of pH on the catalytic activity of the enzyme was studied at 37°C between pH 4.0-10.0. The pH curve displayed maximal activity at pH ranging from 7.5-9.0, as

TABLE II

Hydrolysis of bioactive peptides by rHtrA2

Substrate	Rates of hydrolysis (nmol/µg/min)	Peptide sequence[a]
Dynorphin A_{1-13}	112	YGGFLR↓R↓IRPKLK
Neurotensin	85	ELY↓ENKPR↓RPY↓IL
Angiotensin I	nh	DRVYIHPFHL
Bradykinin	nh	RPPGFSPFR
Oxytocin	nh	CYIQNCPLG

a: cleavage sites determined by mass spectrometry (MS) analysis are indicated by ↓. Assays were carried out in 300 µL of 50 mM Tris-HCl buffer, pH 8.0, at 42°C, using 50 µM of each peptide and 5 nM of rHtrA2. Control samples were identical, except that the enzyme was omitted. After 30 min, the reactions were stopped with TFA (0.1%) and the hydrolysis rates were determined by comparing the peak areas of control samples versus digested samples. The peaks were manually collected and submitted to MS analysis for cleavage bond determinations. nh: no hydrolysis detected.

A

Inhibitors	Velocity (nmol/g^{-1}.min^{-1})	Residual activity (%)
Control	0.0861	100
PMSF (1 mM)	0.0115	13
Aprotinin (4.2U)	nh	0

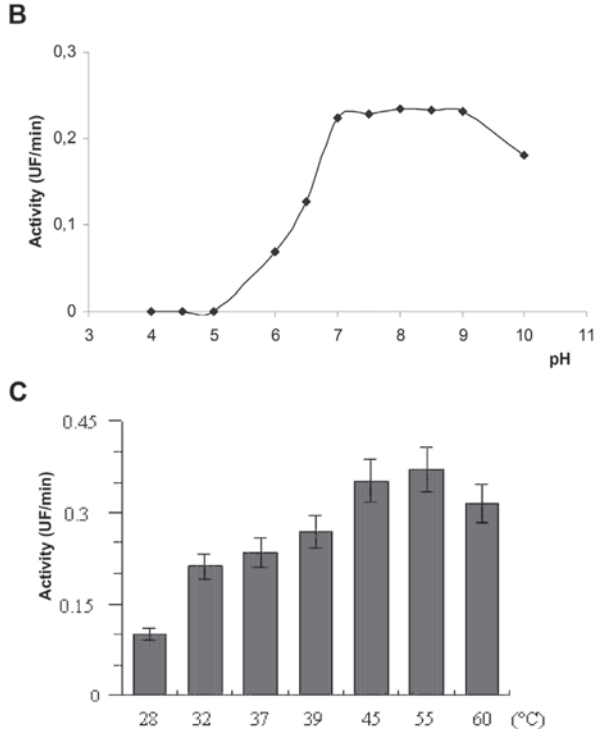

Fig. 3: physicochemical properties of *Mycobacterium leprae* rHtrA2. A: effect of serine protease inhibitors on rHtrA2 activity. Assays were carried out in 500 µL of 50 mM phosphate buffer, pH 8.0, at 37°C and enzymatic activities were measured by fluorometric assays using 5 µM Nα-RR-MCA as substrate. One hundred percent represents HtrA2 hydrolysis of Nα-RR-MCA/min in the absence of an inhibitor. Purified rHtrA2 (2 mU) was incubated in the presence of 1 mM PMSF or 4.2 mU aprotinin under the conditions described above. B: effect of pH upon rHtrA2 enzymatic activity. The pH studies were performed at 37°C in 50 mM sodium citrate buffer (pH 3.0 - 5.3), 50 mM sodium phosphate buffer (pH 5.2-7.5) and 50 mM Tris-HCl buffer (pH 7.3-10). The enzymatic activity was determined as in A: C: effect of temperature on rHtrA2 activity. The experiments were carried out at the stated temperatures using the same buffer and substrate as in A. Enzymatic assays were carried out under the same conditions as in A, except for temperature variation. Data represent the mean of three independent experiments. nh: no hydrolysis detected.

shown in Fig. 3B. The activity of the enzyme was also investigated at various temperatures from 28-60˚C. Fig. 3C shows that *M. leprae* rHtrA2 was active at a broad range of temperatures, reaching maximum activity at 45-55˚C. This preference for high temperatures is a typical feature of proteases belonging to the HtrA family (Clausen et al. 2002, Kim & Kim 2005). Indeed, classical HtrA proteases possess a temperature-dependent functional switch that makes it possible to transform a formerly chaperoned activity to one of protease. At low temperatures, these enzymes exhibit activity of molecular chaperones, while their proteolytic activities rapidly increase between 32-42°C (Skorko-Glonek et al. 2007, 2008). The details of the chaperone activity of *M. leprae* HtrA2 and the question of whether this enzyme displays a similar temperature-dependent functional switch from chaperone to protease activity requires further investigation.

Taken together, our results confirm that ML0176 codes for a serine protease, displaying the physicochemical characteristics typical of an HtrA protein. The possible role of HtrA2 in leprosy remains to be studied and is under investigation in our laboratory. Excitingly, it has recently been reported that HtrA2 deletion in *M. tuberculosis* results in virulence attenuation, with decreased extent of pathology and longer survival times, in a mouse model of tuberculosis (MohamedMohaideen et al. 2008). Moreover, a biochemical analysis of this protein showed serine protease activity towards β-casein and chaperone behaviour. This study also determined the three-dimensional structure of *M. tuberculosis* HtrA2, raising the possibility of designing effective specific inhibitors to this enzyme, which may contribute to novel therapeutic treatment of leprosy and tuberculosis.

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