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Characterization of a leucine aminopeptidase from *Toxoplasma gondii*

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ABSTRACT

The M17 family leucine aminopeptidase (LAP) hydrolyzes amino acids from the N-terminus of peptides. Many LAPs from parasitic protozoa, including *Plasmodium*, *Trypanosoma*, and *Leishmania*, have been intensely investigated because of their crucial roles in parasite biology. In this study, the functional recombinant *Toxoplasma gondii* LAP (rTgLAP) was expressed in *Escherichia coli*, and its enzymatic activity against synthetic substrates for aminopeptidase, as well as cellular localization, was determined. The activity was strongly dependent on metal divalent cations, and was inhibited by bestatin, which is an inhibitor for metalloprotease. Our results indicated that TgLAP is a functional aminopeptidase in the cytoplasm of *T. gondii*.

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa. The parasite infects most species of domestic animals, birds, and humans in both developed and developing countries. It is estimated that up to one-third of the population in the United States is infected with toxoplasmosis, and up to 90% of the populations in other countries are infected with *T. gondii* [1]. In immunocompromised individuals and pregnant women, infection with the parasite can cause severe complications [2].

Leucine aminopeptidases are exopeptidases that catalyze the hydrolysis of leucine residues from the amino termini of protein or peptide substrates [3]. LAPs are one of the major cytosolic aminopeptidases and have been identified in numerous microorganisms, plants, vertebrates and invertebrates [4–7]. They play diverse biological and physiological roles by either degrading peptides or interacting with peptide-dependent signaling [8,9]. Changes in LAP expression pattern or catalytic function resulted in altered peptide activation, leading to changes in tumor cell proliferation, invasion, and angiogenesis.

The LAPs of parasitic organisms such as *Plasmodium*, *Trypanosoma*, and *Leishmania* have been proved to be involved in free amino acid regulation [10–12]. Due to their important roles in parasite biology, many LAPs have recently been investigated as drug targets [12] and vaccine candidates [13,14] in parasitic infections. However, no aminopeptidases in *T. gondii* have received much attention to date. In this study, we cloned and expressed a leucine aminopeptidase of *T. gondii* and determined its cellular localization and enzymatic activity. The data we present here will considerably expand existing knowledge of the protein metabolism of *T. gondii*.

2. Material and methods

2.1. Parasite culture and purification

T. gondii RH strain tachyzoites were maintained in vero cells cultured in a minimum essential medium (MEM, Sigma, USA) supplemented with 8% heat-inactivated fetal bovine serum and 50 μg/ml kanamycin at 37 °C in a 5% CO_2 air environment. For purification of *T. gondii* tachyzoites, parasites and host cells were washed in cold PBS, and the final pellet was resuspended in cold PBS and syringed three times with a 27-gauge needle. The parasites were then filtered through a 5.0 μm pore filter (Millipore, USA), washed twice with PBS, and store at −80 °C until use.

2.2. Cloning of the TgLAP gene

Total RNA of *T. gondii* RH strain was extracted with Trizol reagent (Sigma, USA). Specific primers (forward primer, 5′-ata gaa ttc tat gtc gag ggt tcc tgcg-3′; and reverse primer, 5′-ata gaa ttc cta gtt ctc ttt cgt ttg tgt gc-3′) were designed according to the TgLAP sequence in ToxoDB (accession number, TGME49_090670). The full length of TgLAP cDNA was amplified by using the one-step RT-RNA kit (Takara, Tokyo, Japan) and cloned into the PGEM-T vector (Promega, USA). Subsequently, the gene was sequenced by

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using an automated sequencer (ABI PRISM 3100 Genetic Analyzer, Foster, USA) with amplification primers and additional internal sequencing primers. A computer program, GENETYX version 7.0 (Software Development, Tokyo, Japan), was used for preliminary sequence assembly and analysis. The protein sequence was then sent for analysis with the NCBI/BLAST program. A phylogenetic tree was generated from homologues of the full-length LAP amino acid sequences by the neighbor-joining method, and the confidence of the branching order was verified by making 1000 bootstrap replicates.

2.3. Expression of the recombinant TgLAP in Escherichia coli and production of anti-rTgLAP sera

The cDNA of the *TgLAP* gene was cloned into the prokaryotic expression vector pGEX-4T-3, and the resulting plasmid was transformed into an *E. coli* BL21 strain (Amersham Pharmacia Biotech). Purification of the rTgLAP was performed with Glutathione Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The rTgLAP fused with GST was eluted by 20 mM reduced glutathione (GE Healthcare, Piscataway, USA) and dialyzed against 50 mM Tris–HCl (pH 8.0). The protein concentrations were measured with the Bio-Rad Protein Assay (Bio-RAD Laboratories, Hercules, USA).

2.4. Electrophoresis

SDS-PAGE was carried out on a 12% (w/v) polyacrylamide gel as described by Liao et al. [15]. PAGE under native conditions was performed by using NativePAGE™ Novex® Bis-Tris Gel System and following the instructions of the manufacturer (Invitrogen, Carlsbad, USA). Proteins were stained with Coomassie Brilliant Blue R-250.

2.5. Preparation of mouse sera against TgLAP

Female Jcl:ICR mice (6 weeks old; CLEA Japan Inc., Tokyo, Japan) were immunized intraperitoneally with 100 μg of purified rTgLAP emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, USA) for the first injection. Fifty microgram of the same antigen in Freund's incomplete adjuvant (Difco) was intraperitoneally injected into the mice on days 14 and 28. Sera were collected 14 days after the last immunization.

2.6. Western blotting

The *T. gondii* lysates were separated on 12% SDS-PAGE under reducing conditions, and Western blotting was performed as described by Liao et al. [15].

2.7. Construction of transgenic T. gondii tachyzoites overexpressing TgLAP

A transgenic *T. gondii* RH parasite strain overexpressing the homogeneous TgLAP was generated to further confirm this point. The transfer vector was constructed as follows: the open reading frame of TgLAP was first used to replace the fragment between the NcoI and NheI restrictions of the pHXNTPHA [16]. Subsequently, a sequence housing full-length TgLAP fused with the HA fragment at C-terminal was amplified with two primers (5′-atg tcg agg gtt cct gcg cc-3′ and 5′-gaa gag gct att atc cgc tgt-3′) and then inserted into the EcoRV site of the pDMG vector [17]. The transfection was performed as described previously [17].

2.8. IFAT and confocal laser microscopic observation

Wild-type and the transgenic parasites were plated on HFF grown on 12-mm coverslips (approximately 2×10^5/well) in 24-well plates. After 16–24 h incubation, coverslips were fixed with 3% paraformaldehyde in PBS for 15 min and were then permeabilized with 0.3% Triton X-100 in PBS for 5 min. Mouse anti-rTgLAP polyclonal antibody and anti-HA monoclonal antibody (Covance Research Products, Berkeley, USA) diluted in PBS containing 3% BSA were used as primary antibodies. Goat anti-mouse Alexa 488 antibodies (Molecular Probes) and goat anti-mouse Alexa 594 antibodies (Molecular Probes) were used as secondary antibodies. Coverslips and glass slides were mounted with Mowiol (Calbiochem) and observed under a confocal laser microscope (TCS NT, Leica, Wetzlar, Germany).

2.9. Enzyme assay

The aminopeptidase activity was determined by measuring the rate of liberation of L-leucine from a fluorogenic substrate, L-leucine-4-methyl-coumaryl-7-amide (Leu-MCA, Peptide Institute, Osaka, Japan). The released AMC was measured using a fluorescence micro-plate reader, Fluoroskan Ascent FL (Thermo Electron Corporation, Waltham, USA), with a wavelength pair of 355–460 nm for both emission and excitation. To determine the pH dependent activity, acetate/Tris buffers (50 mM acetic acid and 100 mM Tris–HCl, pH 4–11) containing 1 mM $MnCl_2$ and rTgLAP and 0.1 mM Leu-MCA at 37 °C were used. Cation sensitivity was investigated by assaying the rTgLAP activity after pre-incubating the enzyme at 37 °C for 30 min in 50 mM Tris–HCl (pH 8.0) containing a metal chloride (Sigma–Aldrich, St Louis, USA). To determine the inhibitory efficacy of bestatin to the enzymatic activity of rTgLAP, the enzyme was pre-incubated with bestatin for 30 min at 37 °C before the substrate was added to measure the residual activity. The relative inhibition levels of the rTgLAP were assessed using bestatin at various concentrations.

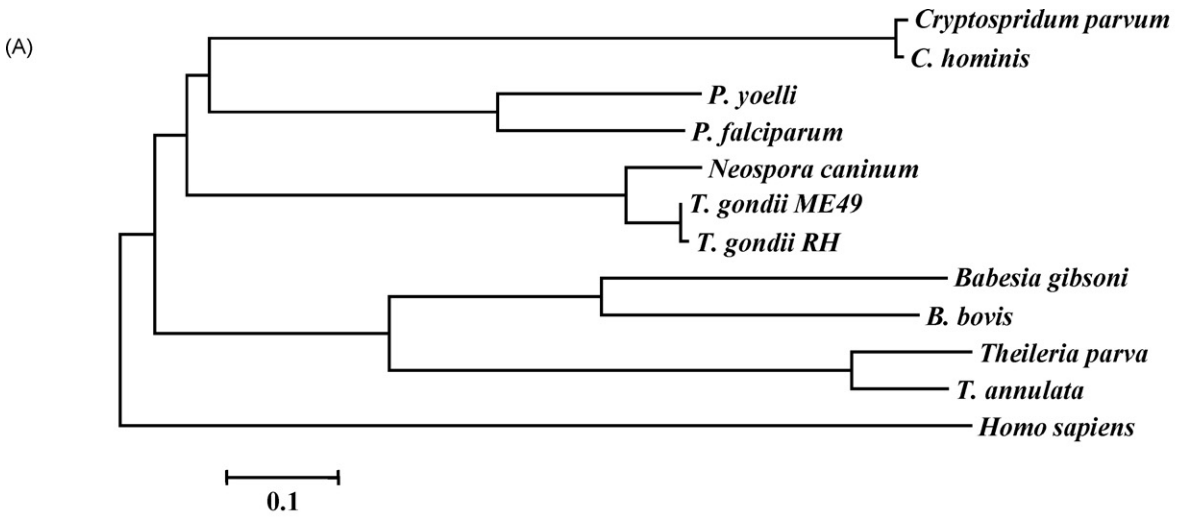
2.10. Enzyme kinetics

The K_m (Michaelis constant) and V_{max} (maximum velocity) values of rTgLAP were determined by incubating the enzyme in the reaction mixture in the presence of increasing concentrations of various fluorogenic substrates (Peptide Institute) at 37 °C. The initial velocity was calculated from the slope of the linear range of fluorescence versus the time curve. The K_m and V_{max} values were recorded with their standard errors derived from three independent experiments.

3. Results and discussion

3.1. Identification of the TgLAP gene

The full-length TgLAP cDNA was intermediate in length among the LAPs of apicomplexan parasites. Two functional domains, a less conserved N-terminal domain (residues 48–208) and a more conserved catalytic C-terminal domain (residues 245–551), were identified using the Pfam protein search algorithm of the SMART program (http://smart.embl-heidelberg.de/). Zn-binding sites (residues 327, 332, 351, 411, 413, and 415) and substrate-binding/catalytic sites (residues 327, 332, 339, 351, 411, 413, and 440) were identified in TgLAP by the BLAST program. M17 aminopeptidases are reported to be homohexameric enzymes [12]. In the amino acid sequence of TgLAP, the interface sites between two trimers were also identified, which indicated that the native TgLAP could exist as homohexamers in the parasite as well. Although the identity of these sites was relatively low, their positions are very similar, especially in the conserved C-terminal. We



(B)

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T. gondii RH    ----------------------------------------------MSRVPAPLTVVKTDPTAIPFVGCHPAKKVRMEAVDISEAEKYSGDLIVFSVLAPGCF 57
P. falciparum   MYFSSLCKFLPISEKEKIYLNIVKKRFCKSNIYYNNNNNNIINYNKRGLKFYPFCNNLKKNINFVNINNKKGINFHSINKERKMASEVPQVVSLDPTSIPIEYNT 105
C. hominis      ----------------------------------------------------------------------MVNPIHPECIPMSEFNESLKFEFS 24
T. annulata     ----------------------------------------------------------------------MDVNEYPYTGFPGESVKFHF-ESPDNTK 27
B. bovis        ----------------------------------------------------------------------MEKILEFPSTDSPVNGVEISLLQTSDAPG 29

T. gondii RH    HDAPNASQPQKGPIALPELAQKFDDAVGQGMLKDAVEAADFKAKMGSQVFVRLPSKSAPAVKHLAALGFGSIAEVTPSAVTKAAAAFASMLLRGSGEKAKTVAVV 162
P. falciparum   PIHDIKVQVYDIKGGCNVEEGLTIFLVNNPGKENGPVKISSKVNDKNVSEFLKDENMEKFNVKLGTSKHFYMFNDNKNSVAVGYVGCGSVADLSEADMKRVVLSL 210
C. hominis      VLKDKYDESLKGDCLVLCVPSKDIMQSIPSFYESLDTILNGILKDIIETANQESSLKDENNTIHVNLPPKYPFKYLMLISFDENKNTSPVFFNKMITQMNEFQKK 129
T. annulata     LEDFNFPKGSLGVFHLAFSSSKKSDPVDNEDYSLESLDFFPSSFYQKNSALPHLAKNNKFSASLCDKLSTVSTR-EDGELLVESVVGCGNKLELLTTDCLTLAAT 131
B. bovis        SDVIKGYQGGVAIFHLCFTATTPSTD-KKPGFDQSKFDVFPTGFITNSDGVKAVIGYSGFSGK-CGDLVEYFDRCDDGSLLSEAVVGCGPHKEFCASDAYTVSKT 132

T. gondii RH    LPTCQKVCPSAEGQRKTREMKERVVTSFLETLLVELQPDLRFKGDREDGKHSGPQVEKLTLFTDDVEAVNR-AIARAKVVAPGVYFAQELVNAPANYCNPVTLAR 266
P. falciparum   VTMLHDNKLSKLTVVFEINVDKNLFRFFLETLFYEYMTDERFKST--DKNVNMEYIKHLGVYINNADTYKE-EVEKARVYYFGTYYASQLIAAPSNYCNPVSLSN 312
C. hominis      NKNIQSFTVVLGFDNVVSSDVMYFCTQFPIDNTIEKRFKGNIVGSEPKKPTKKIERIHFVLRSEDTIKDCNSRVLDAQFVSRGLLFARDLTSAPPNYCDPVNMAK 234
T. annulata     MASVLTSNKLKVAALLLLDFTEQYVELLLTNLLVQLCPDNRFKKD-NKNEFYLENLFVFNKHSKNLAHLESVFFLKVKMYSESMNMAKELTTAPPNYANTVTVSE 235
B. bovis        IAKAISTHQCKHAIFMVGGLEPVLIESLVIGSLNCISVDRRFKKD-TNVEYYLESLHVIADAISELNTFQT----RCKAFVRGMHTTRELVTAPANYANTESIAG 232

T gondii RH     AAVEMAKEAGLEAEVLQQDDIEKLKMGCYLAVAKGSLFPPQFIHLTYKS-SNPKRRVAFVGKGICFDAGGYNLKTG-GAQIELMKFDMGGAAAVLGAARALGELK 369
P. falciparum   AAVELAQKLNLEYKILGVKELEELKMGAYLSVGKGSMYPNKFIHLTYKSKGDVKKKIALVGKGITFDSGGYNLKAAPGSMIDLMKFDMSGCAAVLGCAYCVGTLK 417
C. hominis      EVISMAKSVGLEGKILQTKECEELKMGAFLAVAQGSKSPAQFVHLTYKPKGEIKKRIALVGKGITMDTGGYNIKHQ---MIHFMKGDMGGAAAVFGTALSVGLIK 336
T. annulata     FLKKMLSSVGLKVKVLDYDDCKKLKMFCYLSVCQGSKFPPKFLHAVYKPDGEVKKTLAFVGKGITFDAGGYNVKNS-STMIHYMKLDMGGFATVFSAAYALGKLK 339
B. bovis        FLQNRLTGLGLEVRIIEEDECRALGMGAYLAVGQGSQYPPKFLHAIYRSSVSVKTKIALVGKGIMFDTGGLNIKSA-ASEIELMKFDMGGMSTVFGAAETIAALK 336

T. gondii RH    PENVEVHFLVAACENMISAKSYRPGDVITASNGKTVEVGNTDAEGRLTLADALVYAEKTVKADTIVDVATLTGAVIVALGYKYAGLWSNKECLASSILKSAENSG 474
P. falciparum   PENVEIHFLSAVCENMVSKNSYRPGDIITASNGKTIEVGNTDAEGRLTLADALVYAEK-LGVDYIVDIATLTGAMLYSLGTSYAGVFGNNEELINKILNSSKTSN 521
C. hominis      PENIEVHFISAICDNLVSRDAYLPGCIITASNGKTIEVGNTDAEGRLTLADALVYACN-LKVETVIDLATLTGANYKLFEGRYASVLGNNDELFQMIQTCGKTVY 440
T. annulata     PKNVELHFVGGLCENMLDANSYRPSDVVTASNGKTVEVLNTDAEGRVTLADSLYYAAK-LDPDYLVDYATLTGAAMTALGVKCAAFYSNDEDLAELYTNSARNSG 443
B. bovis        PHGVEVHFISATCENMAGSNAYRPGDIVTASNGKTIEVINTDAEGRLTLADALVYADK-LGVDYIVDLATLTGACIIALGYQYGGYFVNDESFHQRFQKALSDSG 440

T. gondii RH    EYMWHMPLAKDYAEALDSKCADMNNVGGKGKGGSIIAAVFLNKFIE-QASWAHIDITGTAWDMKTSMATGFGVRTLVQYIMDISTQTKEN                563
P. falciparum   EPVWMLPIINEYRATLNSKYADINNISSSVKASSIVASLFLKEFVQ-NTAWAHIDIAGVSWNFKARKPKGFGVRLLTEFVLNDAL-----              605
C. hominis      EKFWQLPLDPEFQEMVKSEIADLNNTS-EGKAPISTSAAFLCEFVDKNVKYAHIDIAGCS--GMGTFGNGFGVKTLVTIIKELSSKC---            524
T. annulata     ELMWRMPLLKEYKDYLNSKVADYANVNYNGKCGSINGALFLKEFVD-DKKWLHVDFAGPAFDFKNEKGTGFGVLTIVNFVLTFT------             526
B. bovis        ELAWRLPLAREYKEGLESKVADLSNCNYTAKAGTVMAALFLNEFVE-NAKWIHWDIAGTAFDKSTGRGTAYGLRTIVNLVLDMAEE----             525
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Fig. 1. Phylogenetic relationship of TgLAP with other LAPs of apicomplexan parasites. (A) Phylogenetic tree of the TgLAP with other LAPs. The scale at the bottom measures the distance between sequences. Sequences used in this study are as follows: *Cryptospridum parvum* LAP (accession number XP_626197), *C. hominis* LAP (accession number XP_667960), *T. gondii* RH LAP (accession number EEB01321), *P. yoelli* LAP (accession number XP_729735), *P. falciparum* LAP (accession number XP_001348613), *Theileria parva* LAP (accession number XP_764196), *T. annulata* LAP (accession number CAI76586), *Babesia gibsoni* LAP (accession number AB490782), and *B. bovis* LAP (accession number XP_001609968). *Homo sapiens* LAP (accession number AAD17527) was used as an outgroup. *T. gondii* ME49 LAP (accession number TGME49_090670) was from ToxoDB (http://toxodb.org/toxo/) and *Neospora caninum* LAP (accession number NC_LIV_113250) was from GeneDB (http://www.genedb.org/). (B) Alignment of the amino acid sequences of the LAPs of apicomplexan parasites. Conservation between amino acids is indicated by asterisk and dots in the alignment. The conserved C-terminal region is presented in bold. The substrate-binding site residues are marked in red, the interface sites between two trimers are marked in green, and the essential Zn-binding sites are underlined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

assessed the phylogenetic relationship between TgLAP and its closest homologues of apicomplexan protozoan parasites (Fig. 1). The overall sequence identity between the TgLAP and the LAP of *P. falciparum* was 36.1%, and that within the C-terminal domain was 55.5%.

3.2. Polyacrylamide gel electrophoresis of rTgLAP expressed in E. coli

The cDNA of the TgLAP gene was cloned into the prokaryotic expression vector pGEX-4T-3, and the resulting plasmid was transformed into an *E. coli* BL21 strain. An rTgLAP fused with GST at the N-terminal was expressed as expected. The molecular weight of the monomer of rTgLAP was around 85 kDa as observed in SDS-PAGE (Fig. 2A). The polymer of rTgLAP with molecular weight of around 500 kDa was observed in native PAGE analysis (Fig. 2B). The use of G-250 charge-shift in NativePAGE™ gels results in protein resolution based upon protein size allowing accurate size estimation of native proteins and protein complexes [18]. Although the size estimation may have an expected size estimation error of ~15%, this result indicated that the

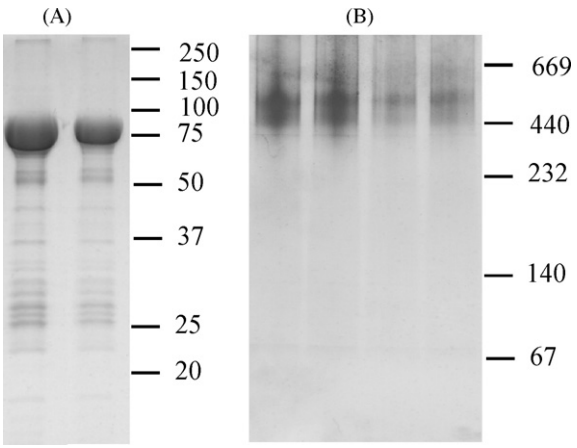

Fig. 2. Electrophoresis of purified rTgLAP. (A) SDS-PAGE of purified rTgLAP. (Lane 1) 10 μg of rTgLAP and (lane 2) 5 μg of rTgLAP. (B) Native PAGE analysis of purified rTgLAP. (Lanes 1 and 2) 10 μg of rTgLAP and (lanes 3 and 4) 5 μg of rTgLAP.

functional form (homohexamer) was present in the purified rTgLAP.

3.3. Western blot analysis of native and HA-tagged TgLAP

A polyclonal anti-rTgLAP serum raised in an ICR mouse was used to identify the native TgLAP in the lysate of *T. gondii* parasites. A specific band with a size of around 60 kDa was detected by Western blotting in the *T. gondii* lysates (Fig. 3A). A transgenic *T. gondii* expressing HA-tagged TgLAP was successfully constructed. As shown in Fig. 3A, an anti-HA monoclonal antibody specifically recognized HA-tagged TgLAP in the transgenic parasites, although an unexpected band could be observed in the blot. Since there was not signal was detected in the control at all, the unexpected band should be degraded product.

3.4. Localization of TgLAP

The specific fluorescence stained by anti-TgLAP polyclonal mouse antibodies under confocal laser scanning microscopy indicated the localization of TgLAP in the cytoplasm of *T. gondii* (Fig. 3B). The cytosolic localization was also confirmed by staining the transgenic parasites with an anti-HA monoclonal antibody (Fig. 3B). Que et al. [19] suggested that cytosolic neutral aminopeptidases were likely involved in the hydrolysis of the dipeptides digested by the TgCPCs in parasitophorous vacuoles (PV). It requires the dipeptides be sent to the cytoplasm where TgLAP localized. However there was no evidence that indicated *T. gondii* tachyzoites could uptake them. Alternatively, another source of the substrates of TgLAP could be the peptides derived from the proteasomal protein degradation pathways.

3.5. Enzymatic activity of rTgLAP

The enzymatic activity of rTgLAP was analyzed against synthesized substrates for aminopeptidase. The protein concentration was chosen in order to obtain the linearity of the reactions (Fig. 4A). Data were fit to the Michaelis–Menten equation using GraphPad Prism version 4.0c (GraphPad Software, San Diego, USA) (Fig. 4B). Activity was noted at around a neutral to slightly alkaline pH range

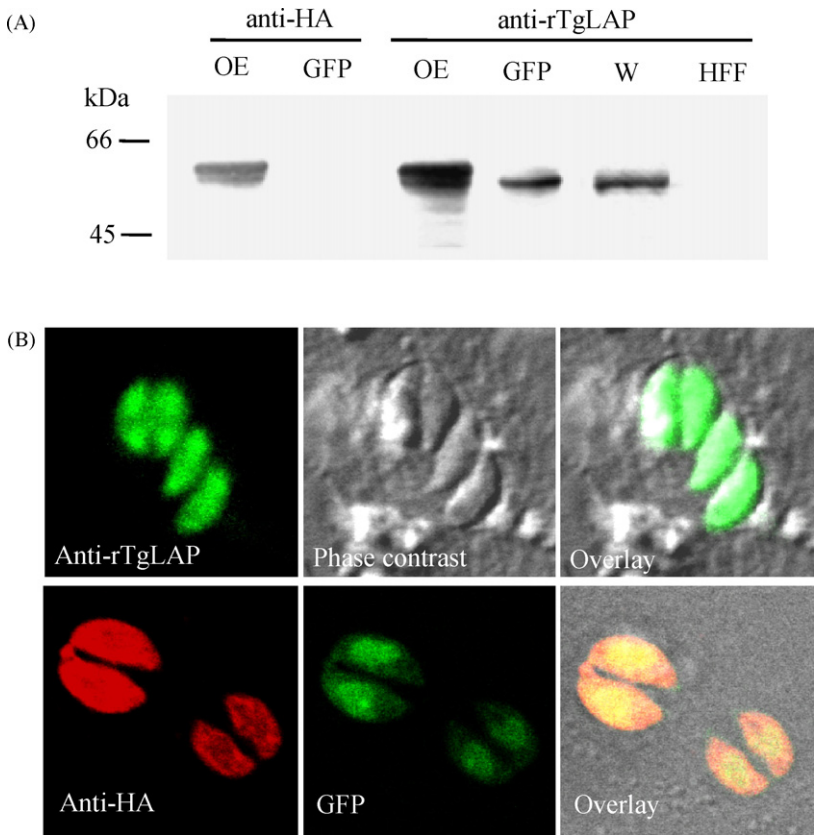

Fig. 3. Western blotting and IFAT of native and HA-tagged TgLAP. (A) Western blot analysis of native and HA-tagged TgLAP. OE, lysates of *T. gondii* RH strain overexpressing TgLAP; GFP, lysates of *T. gondii* RH strain expressing GFP alone; W, lysates of wild-type *T. gondii* RH strain; HFF, lysate of HFF cells. (B) Indirect immunofluorescence detection of the stained TgLAP in cytoplasm. (a) Wild-type RH *T. gondii* stained with anti-rTgLAP polyclonal antibodies; (b) phase contrast of Panel a; (c) an overlay of Panel a on Panel b; (d) transgenic *T. gondii* stained with an anti-HA monoclonal antibody; (e) fluorescence of GFP in transgenic *T. gondii* and (f) an overlay of Panel d on Panel e.

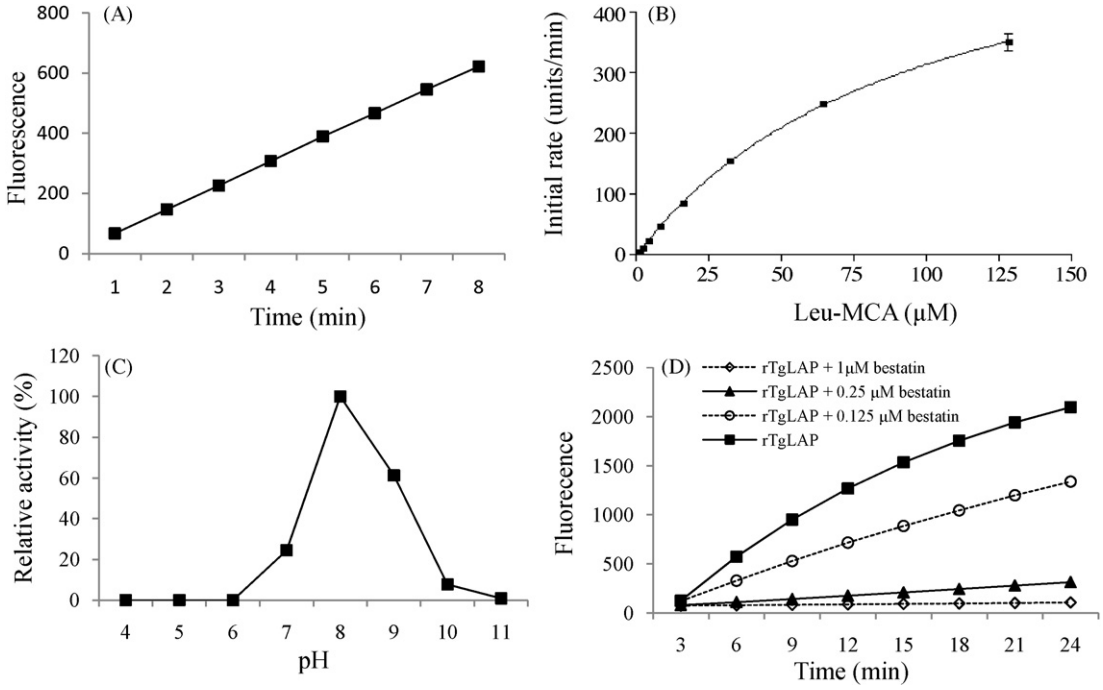

Fig. 4. Enzymatic activity of the rTgLAP. (A) An example of linear reaction of the rTgLAP against synthetic substrates; (B) enzyme assays with the fluorogenic peptide substrate Leu-MCA demonstrate that the rTgLAP exhibits Michaelis–Menten enzymatic kinetics. One unit was defined as picomoles of AMC released per milligram of recombinant protein; (C) pH-dependence of rTgLAP was analyzed against Leu-MCA from pH 4 to 11; (D) inhibition of rTgLAP activity against Leu-MCA by bestatin. Inhibition of the rTgLAP activity was studied using bestatin (Sigma–Aldrich) at final concentrations of 0, 0.125, 0.25, and 1 μM in the reaction mixture. Data points indicate the mean activity ± SD ($n = 3$).

(pH 7–10), and optimum activity was achieved at pH 8.0 and 37 °C (Fig. 4C). The metal cation sensitivity was investigated by assaying the rTgLAP activity after pre-incubating the enzyme at 37 °C for 30 min in 50 mM Tris–HCl (pH 8.0) containing a metal chloride (Sigma–Aldrich, St. Louis, USA). In the absence of metal ion from the reaction buffer, the rTgLAP activity was almost undetectable. However, the activity was markedly activated by the addition of Mn^{2+} or Co^{2+} (Table 1). Therefore, the rTgLAP seems to be purified as an apoenzyme, although the metal contents in the rTgLAP still need further investigations. Similar results were also observed in other reports [4,6]. The *in vitro* inhibition assay of rTgLAP activity using bestatin, which is known as the inhibitor of M1 and M17 cytosolic aminopeptidases [20], is shown in Fig. 4D. The rTgLAP activity was inhibited by bestatin in a dose-dependent manner. Bestatin is a dipeptide analog first discovered as an antibiotic of the bacterium *Streptomyces olivoreticuli*. It has very low toxicity in experimental animals and humans [21,22] and, consequently, has been formulated for the safe therapeutic treatment of certain cancers such as squamous cell carcinoma in humans [23]. These dipeptide analogs will be useful scaffolds on which novel small molecule inhibitors could be designed to potently and selectively inhibit the *T. gondii* M17 family aminopeptidases.

Table 1
Effect of divalent metal ions on rTgLAP activity.

Metal ion	Concentration (mM)	Activity (units/min)[a]
Fe(II)	0.1	0.07 ± 0.1
	1	0.46 ± 0.09
Mg(II)	0.1	0.02 ± 0.22
	1	0.22 ± 0.29
Ca(II)	0.1	0.09 ± 0.03
	1	0.13 ± 0.07
Ni(II)	0.1	0.07 ± 0.01
	1	24.72 ± 1.29
Mn(II)	0.1	109.49 ± 9.22
	1	225.72 ± 14.37
Cu(II)	0.1	0.01 ± 0.08
	1	0.16 ± 0.04
Zn(II)	0.1	0.43 ± 0.05
	1	0.46 ± 0.02
Co(II)	0.1	89.45 ± 8.66
	1	105.2 ± 1.25

[a] One unit was defined as picomoles of AMC released per milligram of recombinant protein. Data represent means ± SD from three independent experiments.

3.6. Substrate specificity of rTgLAP

The enzyme kinetics of rTgLAP against fluorogenic synthetic substrates matched its classification as a member of the M17 leucine aminopeptidase family. Hydrophobic amino acid leucine could be efficiently cleaved by the rTgLAP from the N-terminus of synthetic substrates. The overall catalytic efficiency of rTgLAP to hydrolyze this amino acid, k_{cat}/K_m, was 832 M^{-1} s^{-1} (Table 2). The preference of rTgLAP for other synthesized substrates is slightly different from that of the LAP of *Babesia gibsoni* [4] or *P. falciprum* [12]. In the case of hydrophobic amino acids phenylalanine and

Table 2
Kinetic parameters for the hydrolysis of peptide substrates by rTgLAP.

Substrate	k_{cat} ($\times 10^{-2}$ s^{-1})[a]	K_m (mM)[a]	k_{cat}/K_m (M^{-1} s^{-1})
Leu-MCA	8.89 ± 0.86	0.107 ± 0.013	832
Ala-MCA	3.04 ± 0.58	0.169 ± 0.041	179.83
Arg-MCA	4.05 ± 0.84	0.426 ± 0.108	95.10
Pro-MCA	0.16 ± 0.03	0.124 ± 0.025	13.2
Phe-MCA	0.002 ± 0.0003	0.010 ± 0	2.30

[a] Data represent means ± SD from three independent experiments.

proline, the enzyme's efficiency values (k_{cat}/K_m) were only 2.30 and 13.2 M^{-1} s^{-1}, respectively. For the neutral amino acid alanine, the k_{cat}/K_m value was 179.83 M^{-1} s^{-1}. Similar to the LAP of *B. gibsoni* but not *P. falciparum* LAP, rTgLAP cleaved to the N-terminal basic amino acid, arginine, with comparable efficiency (k_{cat}/K_m = 95.10 M^{-1} s^{-1}). These slight differences among the LAPs of the parasites were probably caused by the requirement for different environments.

In summary, we report here the cloning, genetic analysis, and biochemical characterization of a novel leucine aminopeptidase from *T. gondii*. The optimal amidolytic activity of TgLAP was observed against a fluorogenic synthetic substrate of Leu-MCA at pH 8.0 and 37 °C. In addition, consistent with its classification as a member of the M17 leucyl aminopeptidase family, the activity of TgLAP was enhanced by Mn^{2+} and Co^{2+} at millimolar concentrations and inhibited by the broad spectrum aminopeptidase inhibitor bestatin.

Like all other exopeptidase, TgLAP could release free amino acids from the final step of protein catabolism. The substrates of this aminopeptidase might be peptides from proteasomal protein degradation pathways, or/and possibly the ones degraded in PV by endoproteases and sent to cytoplasma. Parasite proteases are increasingly recognized as potential targets for chemotherapeutic agents. Most of them have been determined to play important roles in parasite biology. However, only cathepsins have been studied in *T. gondii*. In this study, we demonstrated that TgLAP was involved in free amino acid regulation by analyzing the functional recombinant enzyme expressed in *E. coli*.

We believe that the characterization of a functional leucine aminopeptidase will provide important insights into the proteolytic cascades of *T. gondii* and will contribute to subsequent studies and on its biology.

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