Title

Enzymatic characterization of a leucine aminopeptidase from *Toxoplasma gondii*

Running title

Characterization of TgLAP

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Abstract

The M17 family leucine aminopeptidase (LAP) hydrolyze amino acids from the N-terminus of peptides. Many LAPs from parasitic protozoa including *Plasmmodium*, *Trypanosoma* and *Leishmania,* have been intensely investigated because of their crucial roles in parasite biology. In this study, a functional recombinant *Toxoplasma gondii* LAP (rTgLAP) was expressed in *Escherichia coli* and its enzymatic activity against synthetic substrates for aminopeptidase, as well as the cellular localization was determined. Our results indicated that TgLAP is a functional aminopeptidase in the cytoplasma of *T. gondii*.

Keywords: *Toxoplasma gondii*; Leucine aminopeptidase; Enzymatic activity

Text

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, as is 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].

The LAPs of parasitic organisms such as *Plasmodium*, *Trypanosoma* and *Leishmania* have been proved to be involved in free amino acid regulation [3, 4, 5]. Due to the important roles of the LAPs in parasite biology, many of them have been investigated as drug targets [5], and vaccine candidates [6,7] in parasitic infections recently. However, none of aminopeptidases has been received considerable attention to date in *T. gondii*. Tachyzoites of *T. gondii* can invade any nucleated cell and survive intracellularly in a specialized parasitophorous vacuole. Within the vacuole, the tachyzoites derive essential nutrients from the host cell, a process likely to involve parasite proteinases. *T. gondii* cathepsin Cs have been reported to be required for peptide degradation in the parasitophorous vacuole [8]. The dipeptides generated by TgCPCs are likely hydrolyzed to amino acids by cytosolic neutral aminopeptidases. However, there still no evidence to

prove this hypothesis. 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 considerably expand our knowledge of the protein metabolism of *T. gondii*.

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 with by using one-step RT-RNA kit (Takara, Tokyo, Japan) and cloned into PGEM-T vector (Promega, USA). Subsequently, the gene was sequenced by 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. 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/). The protein sequence was then sent to be analyzed with the NCBI/BLAST program. The Zn-binding sites (residue 327, 332, 351, 411, 413, 415) and the substrate binding/catalytic sites (residue 327, 332, 339, 351, 411, 413, 440) were highly conserved between TgLAP and other LAPs. M17 aminopeptidases are reported to be homohexameric enzymes [5, 9]. In the amino acid sequence of TgLAP, the interface sites between two trimers was also identified by BLAST program, which might indicate that the native TgLAP could exist as homohexamers in the parasite as well.

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).

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 in the *T. gondii* lysates by using Western blotting but not in the control (Fig. 1A). The specific fluorescence stained by anti-TgLAP polyclonal mouse antibodies indicated the localization of TgLAP in the cytoplasm of *T. gondii* (Fig. 1B). 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 *Nco*I and *Nhe*I restriction of the pHXNTPHA [10]. Subsequently, a sequence that housing full-length of TgLAP fused with HA fragment 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 *Eco*RV site of pDMG vector [11]. The transfection was performed as described previously [11]. The cytozolic localization could be observed under a confocal laser scanning microscopy (TCS NT, Leica, Wetzlar, Germany) by staining the transgenic parasites with an anti-HA monoclonal antibody (Fig. 1B).

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 protein concentration was chosen in order to obtain the linearity of the reactions. 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. The *Km* (Michaelis constant) and *Vmax* (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 37ºC. Data were fit to the appropriate equation using GraphPad Prism version 4.0c (GraphPad Software, San Diego, USA). The initial velocity was calculated from the slope of the linear range of fluorescence versus the time curve. The *Km* and *Vmax* values were recorded with their standard errors derived from three independent experiments.

The metal cation sensitivity was investigated by assaying the rTgLAP activity after

pre-incubating the enzyme at 37ºC for 30 min in 50 mM Tris-HCl (pH 8.0) containing a metal chloride (Sigma-Aldrich, St. Louis, USA). In the absence of Mn^{2+} from the reaction buffer, the rHlLAP activity was quite low. However, the activity appeared to be markedly activated by adding Mn^{2+} or Co^{2+} (Table 1). At around a neutral to slightly alkaline pH range (pH 7 - 10), the activity could be obtained and the optimum activity was achieved at pH 8.0 and 37ºC (Fig. 2A). The *in vitro* inhibition assay of the rTgLAP activity using bestatin, which is known as the inhibitor of M1 and M17 cytosolic aminopeptidases [12] is shown in Fig. 2B. The rTgLAP activity was inhibited by bestatin in a dose-dependent manner.

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 peptides. The overall catalytic efficiency of rTgLAP to hydrolyze this amino acid, *k*cat/*Km*, was 832 M^{-1} s^{-1} (*k*cat = 88.87 ± 8.62 s^{-1}; *Km* = 0.107 ± 0.013 mM). the preference of rTgLAP to other synthesized substrate is slight different with the LAP of *Babesia gibsoni* [13] or *P. falciprum* [5]. In the case of hydrophobic amino acid phenylalanine, proline, the enzyme's efficiency, *k*cat/*Km*, were only 2.30 M^{-1} s^{-1} (*k*cat = 0.02 ±0 .003 s^{-1}; *Km* = 0.010 ± 0 mM) and 13.2 M^{-1} s^{-1} (*k*cat = 1.64 ± 0.27 s^{-1}; *Km* = 0.124 ± 0.025 mM) respectively. For the neutral amino acid alanine, the *k*cat/*Km* values were 179.83 $M^{-1}s^{-1}$ (*k*cat = 30.40 ± 5.76 s^{-1}; *Km,* 0.169 ± 0.041 mM). Similar with the LAP of *B. gibsoni* but not with *P. falciparum* LAP, rTgLAP could cleave N-terminal basic amino acid, arginine with comparable efficiency (*k*cat/*Km* = 95.10 $M^{-1}s^{-1}$; *k*cat = 40.50 ± 8.41 s^{-1}; *Km* = 0.426 ± 0.108 mM). These slight differences among LAPs of these parasites were probably caused by the requirement for living in quite different environment.

Parasite proteases are increasingly recognized ad potential targets for chemotherapeutic agents. Most of them have been determined to play important roles in parasite biology. However, only cathepsins have been studied. Aminopeptidases are the proteases that catalyze the hydrolysis of amino acid from the amino end of polypeptide. LAPs, a family of aminopeptidase, have been identified in numerous microorganisms, plants, vertebrates, and invertebrates [14, 15, 16]. In this

study, we report that a leucine aminopeptdase of *T. gondii* involved in free amino acid regulation. 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 on its biology.

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Figure legends:

Fig. 1. A, Western blot analysis of recombinant the *T. gondii*. 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 cytoplasma. Wild type and recombinant 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 permeabilised 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 respectively. Goat anti-mouse Alexa 488 antibodies (Molecular Probes) and goat anti-mouse Alexa 594 antibodies (Molecular Probes) were used as secondary antibodies respectively. Coverslips and glass slides were mounted with Mowiol (Calbiochem) and observed under a microscope. 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*; f, an overlay of Panel d on Panel e.

Fig. 2. A, the pH-dependence of the rTgLAP was analyzed against I-Leu-MCA from pH 4 to 11. To determine the pH-dependent activity, acetate/Tris buffers (50 mM acetic acid and 100 mM Tris-HCl) containing 1 mM $MnCl_2$ and rTgLAP and 0.1 mM Leu-MCA at 37ºC were used; B, inhibition of the rTgLAP activity against I-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. The enzyme was pre-incubated with bestatin for 30 min at 37ºC before adding the substrate to measure the residual activity. The relative inhibition levels of the rTgLAP were assessed using bestatin at various concentrations. The activity is presented as fluorescence units/min.

Data points indicate the mean activity ± SD (n = 3).

Table 1. Effect of divalent metal ions on rTgLAP acitivity.

* Data represent means ± S.D. from 3 independent experiments.