# Characterization of a leucine aminopeptidase of *Babesia gibsoni*

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SUMMARY

Peptidases of parasitic protozoa are currently under intense investigation in order to identify novel virulence factors, drug targets, and vaccine candidates, except in *Babesia*. Leucine aminopeptidases in protozoa, such as *Plasmodium* and *Leishmania*, have been identified to be involved in free amino acid regulation. We report here the molecular and enzymatic characterization, as well as the localization of a leucine aminopeptidase, a member of the M17 cytosolic aminopeptidase family, from *B. gibsoni* (BgLAP). A functional recombinant BgLAP (rBgLAP) expressed in *Escherichia coli* efficiently hydrolysed synthetic substrates for aminopeptidase, a leucine substrate. Enzyme activity of the rBgLAP was found to be optimum at pH 8·0 and at 37 °C. The substrate profile was slightly different from its homologue in *P. falciprum*. The activity was also strongly dependent on metal divalent cations, and was inhibited by bestatin, which is a specific inhibitor for metalloprotease. These results indicated that BgLAP played an important role in free amino acid regulation.

Key words: *Babesia gibsoni*, leucine aminopeptidase, enzymatic activity.

## INTRODUCTION

*Babesia gibsoni* is an intra-erythrocytic, tick-borne protozoan parasite that infects dogs. The infection caused by *B. gibsoni* is considered to be epidemic in many areas worldwide and, recently, presents frequently in dogs. Reproduction in both ticks and dogs is required for this parasite to maintain the transmission cycles. When transmitted to dogs by ticks, *B. gibsoni* parasitizes in the red blood cells and causes many of the clinical symptoms of babesiosis, including remittent fever, progressive anaemia, and haemoglobinuria, and sometimes death (Muhlnickel *et al*. 2002; Boozer and MacIntyre, 2003; Ikadai *et al*. 2004).

Aminopeptidases, which catalyse the cleavage of amino acids from the amino-termini of proteins or peptide substrates (Taylor, 1993), play an important role in several physiological processes. Some of them take part in metabolic pathway regulation, cell-cycle control, and selective protein degradation (Brownlees and Williams, 1993). The aminopeptidases of parasitic protozoans are becoming increasingly important as virulence factors, drug targets, and vaccine candidates in parasitic infections recently, e.g. the vaccination of sheep with *Fasciola hepatica* leucine aminopeptidase (LAP) (Acosta *et al*. 2008; Marcilla *et al*. 2008) and the direct application of specific aminopeptidase inhibitors to control infection by *P. falciparum* (Stack *et al*. 2007) and *Trypanosoma brucei* (Knowles, 1993). The LAPs from *Plasmodium* and *Leishmania* have been identified to be involved in free amino acid regulation (Morty and Morehead, 2002; Stack *et al*. 2007). Despite these observations, no aminopeptidase has ever been studied in any *Babesia* genus protozoan parasites. Therefore, we isolated a novel leucine aminopeptidase of *B. gibsoni* (BgLAP) from an expressed sequence tag (EST) database and report here the cloning, genetic analysis, and biochemical characterization of this enzyme. In addition to providing important new data on the enzymology of this family of peptidases, the data we present here considerably expand our knowledge of the peptidolytic capacity of *Babesia*.

## MATERIALS AND METHODS

### *Parasite and EST database*

*B. gibsoni* NRCPD strain parasites (Fukumoto *et al*. 2001) were maintained in a splenectomized beagle. Total RNA was prepared from dog erythrocytes

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infected with this *B. gibsoni* strain by acid guanidinium thiocyanate-phenol-chloroform extraction methods (Chomczynski and Sacchi, 1987), and the full-length cDNA library was made by using the vector-capping method (Kato *et al.* 2005). Briefly, the cDNA was synthesized by using the G-capping method using 5 $\mu$g total RNA, ligated into the pGCAP1 vector, and then transformed into electrocompetent *Escherichia coli* DH12S cells (Invitrogen, USA). ESTs were constructed by random partial sequencing of the 5′-terminus of 10 000 cDNA clones from the cDNA library (Aboge *et al.* 2008).

### *Analysis and cloning of the cDNA encoding the BgLAP*

The full length cDNA of BgLAP was sequenced 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 to be analysed with the NCBI/BLAST program. Functional domains were identified using the Pfam protein search algorithm of the SMART program (http://smart.embl-heidelberg.de/). 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 (http://align.genome.jp/clustalw/).

### *Expression and purification of rBgLAP*

The cDNA fragment of BgLAP without a signal peptide was inserted into *E. coli* expression vector pGEX-4T-3 (Amersham Pharmacia Biotech, Piscataway, USA). The resulting plasmid was designated as pGEX-4T-3/BgLAP after it was identified by restriction enzyme analysis and sequencing. The recombinant protein fused with a glutathione *S*-transferase (GST) tag was expressed in the *E. coli* BL21 strain according to the manufacturer's instructions (Amersham Pharmacia Biotech). Purification of rBgLAP was performed with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The rBgLAP fused with GST was eluted by 20 mM reduced glutathione (GE Healthcare, Piscataway, USA) and dialysed against 50 mM Tris–HCl (pH 8·0). Thrombin (50 U, GE Healthcare) was added into the glutathione–Sepharose 4B, to which rBgLAP was bound for the removal of the GST tag. After cleavage, the supernatant containing rBgLAP without the GST tag was applied to a HiTrap benzamidine FF column (GE Healthcare Bio-Sciences KK, NJ, USA) to eliminate the contaminated thrombin according to the manufacturer's protocol. The purified recombinant proteins were applied to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentrations were measured with the Bio-Rad Protein Assay (Bio-RAD Laboratories, Hercules, USA).

### *Preparation of mouse sera against BgLAP*

ICR mice (6 weeks old) were immunized intraperitoneally with 0.2 ml (500 $\mu$g/ml in PBS) of purified rBgLAP emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, USA) for the first injection. Fifty $\mu$g 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.

### *IFAT and confocal laser microscopic observation*

A thin blood smear prepared with *B. gibsoni*-infected red blood cells was fixed with acetone at −20 °C for 30 min. The anti-rBgLAP serum raised in a mouse was applied as the primary antibody on the fixed smear and incubated for 30 min at 37 °C. After washing 3 times with PBS, Alexa-Fluor® 488-conjugated goat anti-mouse immunoglobulin G (IgG) (Molecular Probes, Eugene, USA) was subsequently applied as a secondary antibody and incubated for another 30 min at 37 °C. The slide was washed 3 times with phosphate-buffered saline (PBS) and incubated with 6·25 $\mu$g/ml propidium iodide (PI) (Wako, Osaka, Japan) containing 50 $\mu$g/ml RNase A (Qiagen, Gaithersburg, USA) for 10 min at 37 °C. After washing twice with PBS, the glass slides were mounted by adding 200 $\mu$l of a 50% glycerol-PBS (v/v) solution and covering with a glass cover-slip. The slides were examined under a confocal laser-scanning microscope (TCS NT, Leica, Wetzlar, Germany).

### *Western blotting*

The erythrocytes of a *B. gibsoni*-infected dog were washed 3 times with PBS, and lysed with 0·83% ammonium chloride at 37 °C for 10 min, respectively. The merozoites were concentrated by centrifugation at 20 000 **g** for 10 min. The erythrocytes collected from a normal dog were conducted with the same procedure as the control. After sonication for 2 min, the samples were subjected to SDS-PAGE. The gel was electrically transferred to a nitrocellulose membrane, and Western blotting was carried out as described previously (Jia *et al.* 2008).

### *Enzyme assay*

The aminopeptidase activity was determined by measuring the rate of liberation of L-leucine from

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A

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B. gibsoni  MKVDIIEKLPLTESPAAAVNITKLAAEDLTESLASSAFPAPLEVFHFVFAPKKTEKTNEG 60
B. bovis    --MEKILEFPSTDSPVNGVEISLLQTSDAPGSDVIKGYQGGVAIFHLCFTATTPSTDKKP 58
              :: * ::* *:**. .*:*: * :.* . * . ..: . : :**: *:..... ::
B. gibsoni  DVDPRELGRFPRCFVTNEAGLLAVATSCKFAGKHGEVVEFFATTKDGGFLAEALVGCGKE 120
B. bovis    GFDQSKFDVFPTGFITNSDGVKAVIGYSGFSGKCGDLVEYFDRCDDGSLLSEAVVGCGPH 118
            ..*  ::. **  *:**. *: **   . *:** *::**:*   .**.:*:**:**** .
B. gibsoni  EGFKASDAHSLAQSVAKVVGQRNCKTVVLELDGFEVDVLEAMVVGLLNHLSVDKRFRKDS 180
B. bovis    KEFCASDAYTVSKTIAKAISTHQCKHAIFMVGGLEPVLIESLVIGSLNCISVDRRFKKDT 178
            : * ****::::::::**.:. ::** .:: :.*:*  ::*::*:* ** :***:**:**:
B. gibsoni  KTKYELETVHVVAGALVNVEAFGERCKVLTKAMHVTRELVSAPANYANTVSIANFVTEKY 240
B. bovis    NVEYYLESLHVIADAISELNTFQTRCKAFVRGMHTTRELVTAPANYANTESIAGFLQNRL 238
            :.:* **::**:*.*: ::::*  ***.:.:.**.*****:******** ***.*: ::
B. gibsoni  TKLGLTVKVLKEEECLELGMGSYLGVSQGSLHPPRFLHATYKGEGPTKVKIAFVGKGIMF 300
B. bovis    TGLGLEVRIIEEDECRALGMGAYLAVGQGSQYPPKFLHAIYRSSVSVKTKIALVGKGIMF 298
            * *** *::::*:**  ****:**.*.*** :**:**** *:.. ..*.***:*******
B. gibsoni  DSGGYNIKSASSQIETMKMDMGGMSTIFGVADVLAALKPKGVEVHFISATCENMVDSTAQ 360
B. bovis    DTGGLNIKSAASEIELMKFDMGGMSTVFGAAETIAALKPHGVEVHFISATCENMAGSNAY 358
            *:** *****:*:** **:*******:**.*:.:*****:**************..*.*
B. gibsoni  RPGDIVTASNGKTIEVVNTDAEGRLTLADALVYAEKLEVDYIIDVATLTGGCIIALGYKY 420
B. bovis    RPGDIVTASNGKTIEVINTDAEGRLTLADALVYADKLGVDYIVDLATLTGACIIALGYQY 418
            ****************:*****************:** ****:*:*****.*******:*
B. gibsoni  AAFFTDDEDLNNRFVKALDKAGELAWRLPIAKEYTDCLESKIADLSNTSYTVKCSSIAAT 480
B. bovis    GGYFVNDESFHQRFQKALSDSGELAWRLPLAREYKEGLESKVADLSNCNYTAKAGTVMAA 478
            ..:*.:**.:::** ***..:********:*:**.: ****:***** .**.*..:: *:
B. gibsoni  LFLKEFVKSTKWLHWDIAGTAQDNSSLRGTGYGVRTLVNLVFDLAGES 528
B. bovis    LFLNEFVENAKWIHWDIAGTAFDKSTGRGTAYGLRTIVNLVLDMAEE- 525
            ***:***:.:**:******** *:*: ***.**:**:**:*:*:* *
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B

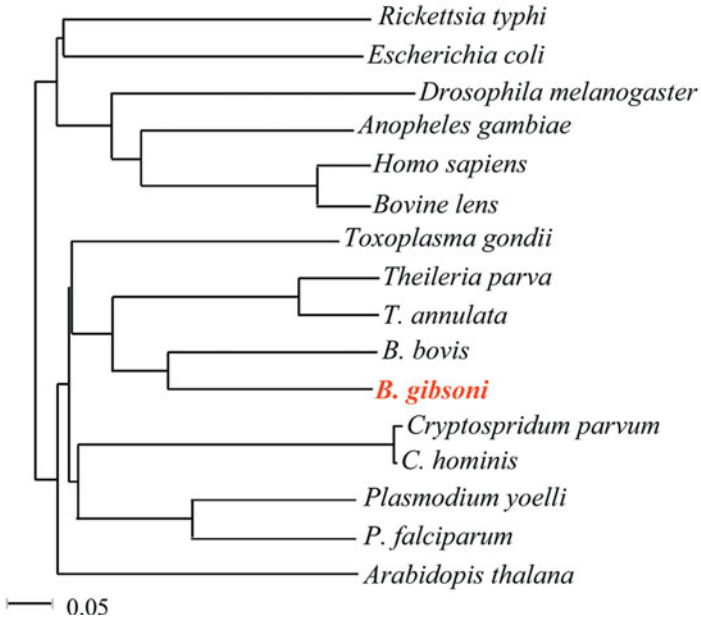


Fig. 1. Phylogenetic relationship of *Babesia gibsoni* M17 leucyl aminopeptidase with other members of the family. (A) Alignment of the amino acid sequences of the LAPs from *B. gibsoni* and *B. bovis*. Conservation between amino acids is indicated by asterisk and dots in the alignment. The conserved C-terminal region is presented in bold. The essential Zn-binding sites are underlined, and substrate-binding site residues are marked in red. (B) Phylogenetic relationship of the M17 cytosolic leucine aminopeptidase family based on pairwise differences. The scale at the bottom measures the distance between sequences. Sequences used in this study were as follows: *Anopheles gambiae* LAP (Accession number EAA06020), *Arabidopsis thaliana* LAP (Accession number NP_194821), *B. bovis* LAP (Accession number XP_001609968), *Bovine lens* LAP (Accession number AAB28170), *Cryptospridum hominis* LAP (Accession number XP_667960), *C. parvum* LAP (Accession number XP_626197), *Drosophila melanogaster* LAP (Accession number AAF50390), *E. coli* O157 LAP (Accession number NP_290893), *Homo sapiens* LAP (Accession number AAD17527), *Plasmodium falciparum* (Accession number XP_001348613), *P. yoelli* LAP (Accession number XP_729735), *Rickettsia typhi* LAP (Accession number AAU03616), *Theileria annulata* LAP (Accession number CAI76586), *T. parva* LAP (Accession number XP_764196), and *Toxoplasma gondii* LAP (Accession number EEB01321).

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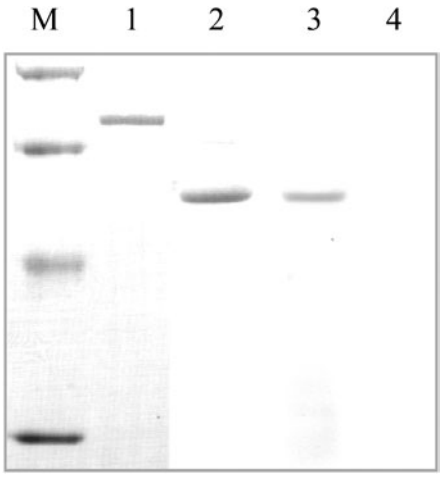


Fig. 2. Molecular mass of the functionally active BgLAP. M, molecular size markers. Lanes 1 and 2, purification of the rBgLAP fused with GST and the rBgLAP alone, respectively were monitored by 12% reducing SDS-PAGE. Lanes 3 and 4, Western blot analysis of the lysates of red blood cells and normal cells, respectively. The blot was stained with a polyclonal anti-rBgLAP serum raised in a mouse.

a fluorogenic substrate, L-leucine-4-methyl-coumaryl-7-amide (Leu-MCA, Peptide Institute, Osaka, Japan). The initial activity rate was defined as the nmol of 7-amino-4-methyl-coumarin (AMC) released by the hydrolysis of the substrate per min per mg of protein. 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. 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 rBgLAP and 0·1 mM Leu-MCA at 37 °C were used. The metal cation sensitivity was investigated by assaying the rBgLAP 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). Inhibition of the rBgLAP activity was studied using bestatin (Sigma-Aldrich) at final concentrations of 1·56, 12·5 and 50 nM in the reaction mixture. The enzyme was pre-incubated with bestatin for 30 min at 37 °C before adding the substrate to measure the residual activity.

### *Enzyme kinetics*

The *Km* (Michaelis constant) and *Vmax* (maximum velocity) values of rBgLAP were determined by incubating the enzyme in the standard reaction mixture in the presence of increasing concentrations of various fluorogenic substrates (Peptide Institute) at 37 °C. Data were fitted 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 3 independent experiments.

### *Nucleotide sequence Accession number*

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the Accession number of AB490782.

All animal experiments described in this article were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine.

## RESULTS

### *Identification of the BgLAP gene*

BgLAP contained 2 domains, a less conserved N-terminal domain (residues 45–180) and a more conserved catalytic C-terminal domain (residues 215–519). The Zn-binding sites and the substrate binding/catalytic sites were highly conserved in *Babesia* and most other LAPs (Fig. 1A). The overall sequence identity between the BgLAP and the LAP of *B. bovis* was high (54·5%), and that within the C-terminal domain was 68·6%.

We assessed the phylogenetic relationship between BgLAP and its closest homologues of the various prokaryotes, plants, and animals. Figure 1B shows that BgLAP formed a distinct clade with the LAP from *B. bovis*. The *Babesia* LAPs were most closely related to those from other members of the Apicomplexa, including *Theileria parva*, *T. annulata*, *Toxoplasma gondii*, *P. falciparum*, and *P. yoelli*, as well as *Cryptosporidium hominis* and *C. parvum*.

### *Molecular mass of BgLAP*

The cDNA of the BgLAP gene was cloned into the prokaryotic expression vector pGEX-4T-3, and the resulting plasmid was transformed into an *E. coli* BL21 strain. An rBgLAP with a molecular weight of 84 kDa was expressed as a fusion protein with GST, as expected (Fig. 2). A polyclonal anti-rBgLAP serum raised in a mouse was used to identify the native BgLAP in the lysate of *B. gibsoni* merozoites. A specific band of around 57 kDa was detected by Western blotting in the *B. gibsoni*-infected red blood cells but not in the normal cells.

### *Localization of BgLAP on parasites*

A thin blood smear was used to perform IFAT with the mouse anti-rBgLAP serum and observed under a

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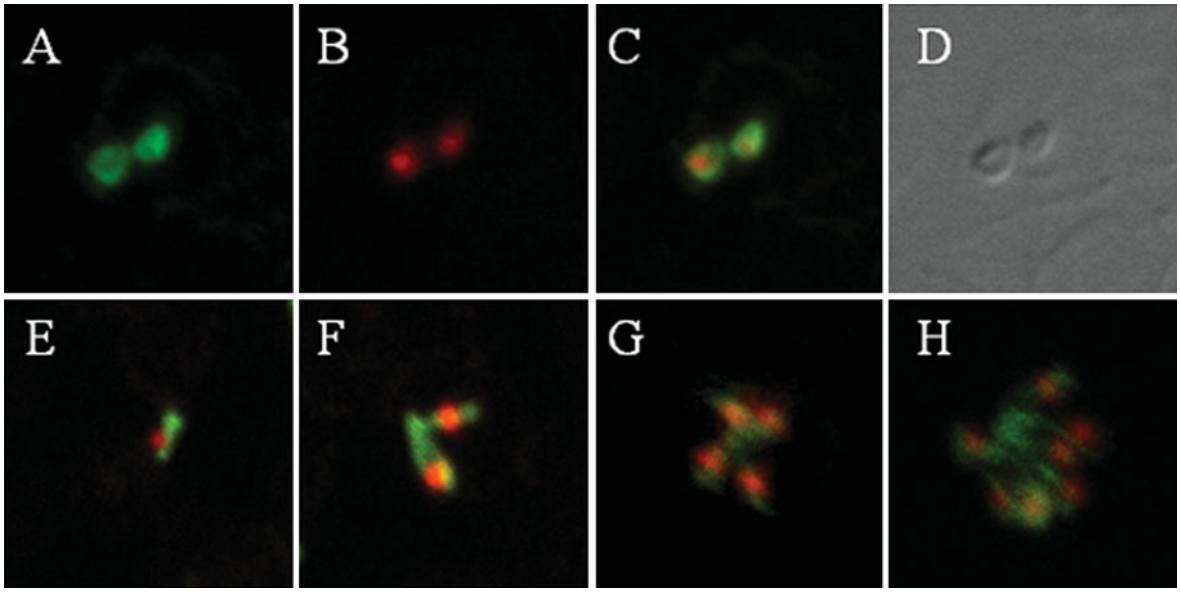


Fig. 3. Observation of native protein recognized by a mouse anti-rBgLAP serum in confocal laser micrographs. (A) Immunofluorescent staining of *Babesia gibsoni* merozoites with a mouse anti-rBgLAP serum. (B) Propidium iodide (PI) staining of *B. gibsoni* merozoite nuclei. (C) Panel A overlaid on Panel B. (D) Phase-contrast images of *B. gibsoni* merozoites. A, B, C, and D were derived from a single section. E, F, G, and H are the images representing single, double, 4, and 8 merozoites stained with anti-rBgLAP serum and PI, respectively.

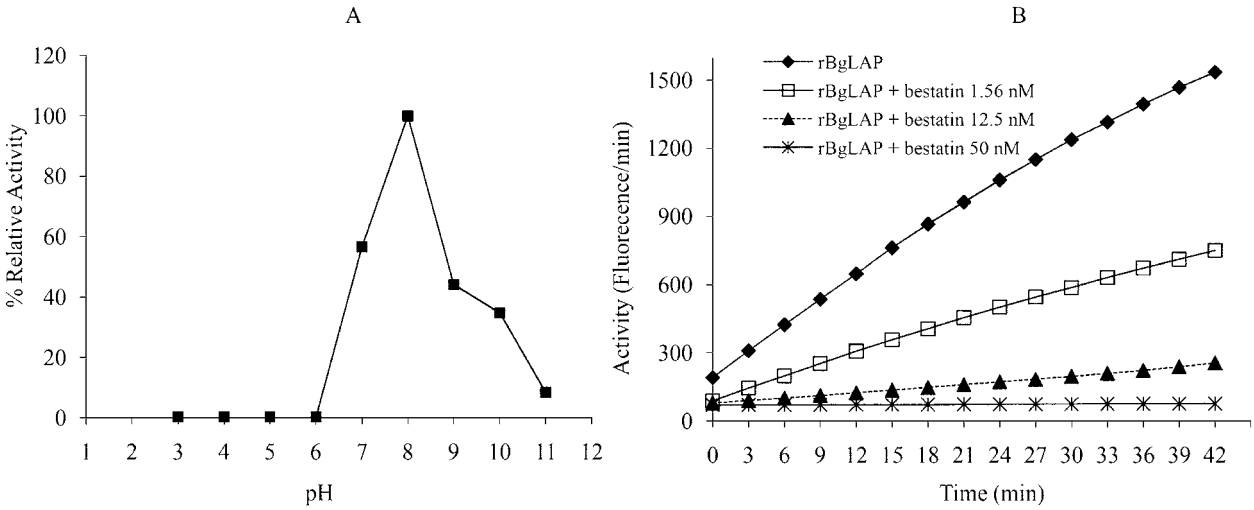


Fig. 4. (A) pH-dependence of the rBgLAP analysed against I-Leu-MCA from pH 4 to pH 11. (B) Inhibition of the rBgLAP activity against I-Leu-MCA by bestatin. The relative inhibition levels of rBgLAP were assessed using bestatin at various concentrations. The activity is presented as fluorescence units/min. Data points indicate the mean activity ± S.D. ($n = 3$).

confocal laser microscope. The specific fluorescence observed agreed with its localization in the cytoplasm of the *B. gibsoni* merozoites (Fig. 3).

### *Amidolytic activity of rBgLAP*

To analyse the enzymatic function of the rBgLAP, we determined the hydrolysing activity using synthetic aminopeptidase substrates. The 84 kDa rBgLAP (*Km*, 0·102 ± 0·023 mM) showed similar catalytic efficiencies against L-Leu-MCA with the 57 kDa rBgLAP (without GST tag) (*Km*, 0·109 ± 0·004). Because the extra procedure and buffer exchange needed for the preparation of the 57 kDa rBgLAP might reduce enzyme stability, the 84 kDa rBgLAP was used for further enzymatic analysis. The activity was obtained at around a neutral to slightly alkaline pH range (pH 7–10), and the optimum activity was achieved at pH 8·0 and 37 °C (Fig. 4A). In the absence of $Mn^{2+}$ in the reaction buffer, the rBgLAP activity was quite low. However, the activity appeared to be markedly activated by adding $Mn^{2+}$. The rBgLAP activity was also enhanced in the presence of several other

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Table 1. Effect of divalent metal ions on the activity of rBgLAP

| Metal ion | Concentration (mM) | Activity (Unit/min)* |
|---|---|---|
| None | — | 0·25 ± 0·011 |
| $Fe^{2+}$ | 0·1 | 0·32 ± 0·003 |
| | 1 | 0·53 ± 0·09 |
| $Ca^{2+}$ | 0·1 | 0·13 ± 0·08 |
| | 1 | 0·20 ± 0·05 |
| $Ni^{2+}$ | 0·1 | 6·37 ± 0·26 |
| | 1 | 10·85 ± 0·10 |
| $Mg^{2+}$ | 0·1 | 1·09 ± 0·41 |
| | 1 | 3·79 ± 1·25 |
| $Co^{2+}$ | 0·1 | 56·76 ± 0·81 |
| | 1 | 36·37 ± 2·70 |
| $Mn^{2+}$ | 0·1 | 137·24 ± 5·39 |
| | 1 | 151·30 ± 4·41 |
| $Zn^{2+}$ | 0·1 | 0·81 ± 0·04 |
| | 1 | 0·33 ± 0·09 |
| $Cu^{2+}$ | 0·1 | 0·079 ± 0·01 |
| | 1 | 0·11 ± 0·03 |

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

divalent metal cations, such as $Co^{2+}$ and $Ni^{2+}$ (Table 1). The *in vitro* inhibition assay of the rBgLAP activity using bestatin is shown in Fig. 4B. Bestatin is a dipeptide originally isolated from filtrates of *Streptomyces olivoretticuli* and is known as the inhibitor of M1 and M17 cytosolic aminopeptidases (Umezawa *et al*. 1976). The rBgLAP activity was inhibited by bestatin in a dose-dependent manner.

### *Substrate specificity of rBgLAP*

The rBgLAP could efficiently cleave the hydrophobic amino acid leucine from the N-terminus of synthetic peptides. Its high affinity for this substrate was reflected in low *Km* and high *k*cat values, resulting in a very high overall catalytic efficiency, *k*cat/*Km*, of 1942 $M^{-1}\,s^{-1}$ (Table 2). The enzyme's preference for this amino acid was significantly greater than that for the hydrophobic amino acid phenylalanine (*k*cat/*Km*, 227 $M^{-1}\,s^{-1}$) and even greater than that for proline (*k*cat/*Km*, 11 $M^{-1}\,s^{-1}$). The substrate with a basic amino acid (Arg) was also cleaved efficiently by rBgLAP (*k*cat/*Km*, 752 $M^{-1}\,s^{-1}$). This result was different from the results obtained with the homologue of the LAP of *P. falciparum*. The enzyme did not cleave substrates with small nonpolar (Gly) and acid (Glu) amino acids.

## DISCUSSION

We report here the cloning, genetic analysis, and biochemical characterization of a novel leucine aminopeptidase from *B. gibsoni*. The full-length BgLAP cDNA was similar in length to the equivalent gene in *B. bovis*, but it was shorter than the LAPs of *Plasmodium* spp. The N- and C-terminal domains, particularly the latter, which houses the catalytic apparatus, are highly conserved between *B. gibsoni* and *B. bovis*. All the residues involved in substrate binding are absolutely conserved among *Babesia* species.

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

| Substrate | $k_{cat} \times 10^3$ | $K_m$ | $k_{cat}/K_m$ |
|---|---|---|---|
| | $S^{-1}$* | mM* | $M^{-1}\,S^{-1}$ |
| I-Leu-MCA | 198·79 ± 45·15 | 0·102 ± 0·023 | 1942 |
| I-Ala-MCA | 8·20 ± 1·66 | 0·095 ± 0·025 | 86 |
| I-Arg-MCA | 37·79 ± 9·05 | 0·189 ± 0·077 | 752 |
| I-Pro-MCA | 2·03 ± 0·65 | 0·050 ± 0·013 | 11 |
| I-Phe-MCA | 24·61 ± 1·16 | 0·108 ± 0·051 | 227 |

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

M17 aminopeptidases are reported to be homohexameric enzymes (Taylor, 1993; Stack *et al*. 2007). In the amino acid sequence of BgLAP, the interface between two trimers was also identified by BLAST program. These data indicated that the native BgLAP could also exist as homohexamers in the parasite.

The optimal amidolytic activity of BgLAP was observed against a fluorogenic synthetic substrate of I-Leu-AMC. Typical Michaelis-Menten enzymatic kinetics and activity were obtained, and optimum activity was achieved 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 BgLAP was enhanced by $Mn^{2+}$ and $Co^{2+}$ at millimolar concentrations and inhibited by the broad-spectrum aminopeptidase inhibitor bestatin. These observations were similar to other M17 leucyl aminopeptidases from *Leishmania* and *Plasmodium* (Morty and Morehead, 2002; Stack *et al*. 2007). The substrate preference of rBgLAP for an N-terminally exposed hydrophobic amino acid was most markedly leucine, but not N-terminal alanine, glycine or glutamic acid. However, of particular interest were our enzyme kinetic data showing the relatively efficient ability of rBgLAP to cleave N-terminal arginine (*k*cat/*Km*, 752 $M^{-1}\,s^{-1}$); this ability might indicate that the LAP of *B. gibsoni* has a broader substrate spectrum than that of *P. falciparum*.

In any living cell, free amino acids are released from the final step of protein catabolism by various aminopeptidases. Thus, the presence of an M17 leucyl aminopeptidase in *Babesia* is not surprising. The LAP in *P. falciparum* was suggested to take part in freeing amino acids from peptides derived from the proteasomal protein degradation pathways or

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those sent to the cytosol from the food vacuole (Dalal and Klemba, 2007). In the case of *Babesia* parasites, BgLAP probably plays a role in the proteasomal protein degradation pathways, since food vacuoles have never been detected in *Babesia* species, and enzymes involved in haemozoin production, i.e. haemoglobin metabolism, are not found in the genomes of *B. bovis* (Potgieter *et al*. 1976; Potgiter and Els, 1977; Lau, 2009).

Many questions remain unanswered regarding the function of the peptidase derived from *B. gibsoni* living in red blood cells. Leucine aminopeptidase is one of the major cytosolic aminopeptidases and has been identified in numerous micro-organisms, plants, vertebrates, and invertebrates (Bartling and Weiler, 1992; Rogi *et al*. 1996; Hatta *et al*. 2006;). However, none of the *Babesia* peptidases has received considerable attention to date. We believe that this is the first identification of an M17 LAP from a *Babesia* parasite. The characterization of a functional leucine aminopeptidase will serve as a solid foundation for subsequent studies on *Babesia* biology.

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