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Title	Expression and characterization of cathepsin B from tsetse (Glossina morsitans morsitans)
Author(s)	Ruttayaporn, Ngasaman; Zhou, Mo; Suganuma, Keisuke et al.
Citation	Japanese Journal of Veterinary Research, 61(4), 137-147
Issue Date	2013-11
DOI	https://doi.org/10.14943/jjvr.61.4.137
Doc URL	https://hdl.handle.net/2115/53708
Type	departmental bulletin paper
File Information	JJVR Vol.61,No.4_P137-147.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Japanese Journal of Veterinary Research 61(4): 137–147, 2013

FULL PAPER

Expression and characterization of cathepsin B from tsetse (*Glossina morsitans morsitans*)

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Received for publication, August 30, 2013; accepted, October 11, 2013

Abstract

Digestive enzymes in tsetse fly midgut are thought to modulate the development of African trypanosome which is a causative agent of trypanosomosis in human and animal. Cathepsin B is induced after the first blood meal ingestion and being higher in trypanosome infected flies. A DNA fragment encoding pro-cathepsin B (930 bp) (Accession No. AF329480_1) was cloned and expressed in *E. coli* and *P. pastoris* protein expression systems. An active recombinant cathepsin B (rGmcathB) produced by *P. pastoris* was migrating from 35 to 45 kDa under reducing condition. rGmcathB exhibited the highest proteolytic activity at pH 4.0 with wide range temperature 25–30°C, also degraded bovine hemoglobin and serum albumin. rGmcathB exhibited hydrolysis preference for Z-Arg-Arg-MCA (K_{cat}/K_M 7.58 $mM^{-1}sec^{-1}$) and bovine hemoglobin (K_{cat}/K_M 3.77×10^3 $mM^{-1}sec^{-1}$). The proteolytic activity of rGmcathB was inhibited by specific cysteine protease inhibitor (E-64) confirmed belonging to papain-like cysteine protease family. These results indicated that rGmcathB shows the activity of cathepsin B and have high affinity with blood protein referring a role in blood meal digestion. In this study, the recombinant protein expressed by *E. coli* expression system was not enzymatically active as shown in the recombinant protein expressed by *P. pastoris* expression system. This finding implies that *P. pastoris* expression system is more suitable for expressing enzymatically active recombinant proteases than *E. coli* expression system.

Key Words: cathepsin B; tsetse fly; enzyme assay

Introduction

Tsetse fly (Diptera: Glossinidae) is a sole vector of African trypanosomes, the causative agent of human African trypanosomosis (HAT) and animal African trypanosomosis (AAT) that has a severe socioeconomic impact in sub-Saharan Africa. African trypanosomes, in particular, *Trypanosoma brucei* and *T. congolense*, undergo complex life cycle development in tsetse fly.

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Bloodstream form trypomastigote differentiates into procyclic form in the tsetse midgut. Then PCF migrates through the proventriculus, and changes to epimastigote form, which is further differentiate into animal infective metacyclic form in either tsetse salivary gland (*T. brucei*) or proboscis (*T. congolense*)[16,19]. In the tsetse midgut, procyclic trypomastigotes must interact with the hostile midgut lumen environment in which proteolytic enzymes and digested products might affect their differentiation and survival. Thus, the midgut is not only the site for synthesis and secretion of digestive enzymes and nutrient absorption, but also is a primary interface between tsetse and trypanosome. Hence, the physiology of blood meal digestion may have substantial influences on trypanosome differentiation and survival in tsetse fly.

Cathepsin B (EC 3.4.22.1) is one of a cysteine proteases, member of papain family contains a conserved active site that is formed by cysteine, histidine and asparagine residues and are inhibited by mercurial compound. This enzyme is the most abundant cathepsin in mammal and invertebrate such as marine shrimp, parasite and insects including tsetse fly[3,24,26,28]. Cathepsin B exhibits an endopeptidase activity which cleave internal peptide bonds, but some exceptionally also display exopeptidase activity. The mammalian cathepsin B involves in protein degradation in lysosome[7] and also participates various physiological processes such as programmed cell death, antigen processing, hormone activation and bone turnover[17,32]. While in parasite, cathepsin B proposed to play several roles in parasitism, including encystment, migration, feeding and immune evasion[23]. Insect cathepsin B participates in many physiological processes such as programmed cell death of the fat body during metamorphosis[14,22,29,31] and intracellular protein degradation during embryogenesis[5,8,21,30,33]. Cysteine proteases, including cathepsin B, were identified in several insect species such as silkworm[11,27], western corn rootworm[12], *Aedes aegypti*[8] as well as in tsetse fly[28]. The previous study reported that the expression of tsetse cathepsin B was induced within a few hours after blood meal and remained high throughout the blood meal digestion cycle. Thus it was speculated that tsetse cathepsin B might have important roles of tsetse-trypanosome interaction[28]. However, enzymatic activity of tsetse cathepsin B has not yet well analysed. Therefore, this study aims to express enzymatically active tsetse cathepsin B and analyse its enzymatic activity using various substrates.

Materials and Methods

Total RNA isolation and cDNA synthesis: Tsetse flies (*Glossina morsitans morsitans*) were kindly provided by Prof. Yuichi Chigusa (Dokkyo Medical University), and maintained at 25°C with 50–55% humidity in National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine. The male tsetse flies (48 hr post blood meal) were anesthetized on ice and soaked in ethanol for 2 min before isolating midgut. Four midguts were pooled to produce total RNA by using TRIzol® reagent (Invitrogen, CA, USA) following the manufacturer's instructions. The RNA concentration was determined by measuring absorbance at 260 nm using Nanodrop system (Thermo Fisher Scientific Inc., MA, USA) further cleaned by using RNase-free DNase, stored at −80°C or immediately used for preparing single stranded cDNA template. The single stranded cDNA was synthesized using a Ready-To-Go T-Primed First-Strand Kit (Amersham Biosciences, NJ, USA) according to the manufacturer's instructions.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The first strand cDNA was amplified using the sense and antisense primers which were designed to amplify coding region of *G. m.*

morsitans cathepsin B (GmcathB) proenzyme as shown in table 1. Ten µl PCR reaction mixture containing 1 µl midgut cDNA template, 2.4 µl PCR master mix, 0.5 µl each of sense and antisense primers and 5.6 µl distilled water, was performed using a thermal cycler (VERITI™ Thermal Cycler, Applied Biosystems, CA, USA). The reaction was subjected to the following conditions: first denature at 94°C for 3 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C 1 min, and final extension at 72°C for 7 min.

Cloning and sequencing: PCR product was separated by agarose gel electrophoresis and extracted using QIAquick Gel Extraction Kit (QIAGEN, MD, USA), cloned into pCR®2.1 vector (Invitrogen, CA, USA), transformed into *E. coli* DH5α. Positive clones were selected by colony PCR. Plasmid clones containing GmcathB gene were extracted using the QIAGEN DNA purification kit (QIAGEN, MD, USA) followed by digestion with restriction enzymes to confirm the corrected insert size. Nucleotide sequences of the cloned gene were determined using M13 sequencing primers with BigDye Terimnator v1.1 cycle sequencing kit (Applied Biosystems, CA, USA). The amino acid sequences were deduced using the GENETYX-WIN DNA analysis software system (GENETYX Co., Tokyo, Japan). Homology search of nucleotide and amino acid sequences were performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Expression of recombinant GmcathB in Escherichia coli: Cloned GmcathB gene was excised by using restriction enzymes, *Eco* RI and *Xho* I, and isolated from electrophoresis gel using QIAquick gel purification kit (QIAGEN, MD, USA). The fragment was sub-cloned into pGEX-4T-1 protein expression vector (GE Healthcare, Little Chalfont, UK), transformed to *E. coli* BL21. Correct insertion of GmcathB gene into the vector plasmid was confirmed by colony PCR and restriction enzyme digestion. *E. coli* containing the recombinant plasmid was grown in LB medium with appropriate antibiotic until OD_{600nm} reach between 0.5 and 0.8. Recombinant protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Insoluble GST fusion proteins were lysed by 2% sarkosyl-TNE buffer, and purified by using a Glutathione Sepharose 4B (GE Healthcare, Little Chalfont, UK) following the manufacturer's instruction. The concentration of purified protein was checked by Nanodrop at 280 nm absorbance and confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Expression of recombinant GmcathB in Pichia pastoris: PCR amplified GmcathB gene was digested by *Mly* I and *Kpn* I and the *P. pastoris* expression vector, pPinkα-HC (Invitrogen, CA, USA), was digested with *Stu* I and *Kpn* I making the 5' blunt end. Digested vectors and gene fragments were used for constructing pPinkα-HC-GmcathB, transformed into *E. coli* DH5α and culture on LB agar containing ampicillin (50 µg/ml). The constructs were verified by colony PCR, restriction enzyme analysis and sequencing. Recombinant plasmids (5–10 µg) were linearized

Table 1. The primer set for RT-PCR

Expression system	Sequence (5'-3')	Target sequence (bp)	Location	Restriction Enzyme
E. coli	**GAATTC** CAT GGA GAC GAT ATA TTG	930	142-159	*Eco* RI
	CTCGAG TTA GCA GTG GTC CTT GCC		1057-1071	*Xho* I
P. pastoris	**GAGTCGACTC** CAT GGA GAC GAT ATA	930	142-156	*Mly* I
	GGTACC TTA GCA GTG GTC CTT GCC		1057-1071	*Kpn* I

Restriction enzyme sites are highlighted by bold. The underline indicates stop codon.

by *Spe* I and transfected into *P. pastoris* PichiaPink strain by electroporation at 1.5 kV, 25 μF for 5 msec. Yeasts were incubated at 24–30°C on *Pichia* adenine dropout (PAD) selective agar for 3–5 days until distinct white colony are formed. The composition of gene into *P. pastoris* genome was confirmed by PCR using a combination of the 5' α-factor or 3' *CYC* 1 primer and a primer that hybridized within the inserted genes. The transformed *P. pastoris* were inoculated into 10 ml of buffered glycerol-complex medium (BMGY), and allow to grow the cell at 30°C with 250 rpm agitation until the OD_{600nm} reach 2–6. Cells were harvested at 1,500 xg for 5 min at room temperature, and suspended with buffered methanol-complex medium (BMMY) at 1/5 of volume of the original culture in order to induce protein expression. The supernatant from 4-day culture containing secreted recombinant protein was dialyzed in PBS (Phosphate buffered saline), concentrated by subject to Amicon ultra centrifugal filter tube (Amicon® Ultra, MA, USA). The BCA protein assay was used to protein quantification (BCA Protein Assay Kit, PIERCE Chemical Company, IL, USA).

Polyclonal antibody production: Antibody against rGmcathB was raised by mixing 100 μg of recombinant GST-fused protein in equal volume of TiterMax Gold adjuvant (CytRx Co., CA, USA) and intraperitoneally injected into mice (female BALB/c, 7 weeks old) with two subsequent boosts at 2 weeks interval. Immune sera were obtained from the mice at 10 days after the 3rd immunization after confirmation of high antibody titre against rGmcathB by ELISA.

Characterization of proteolytic activity: The proteolytic activity was determined by using fluorogenic casein (EnzCheck® Protease Assay Kits-Green Fluorescent, Invitrogen, CA, USA). Briefly, 100 μl of rGmcathB (6 μg/ml) was mixed with 100 μl of 1% v/v BODIPY FL casein (Invitrogen, CA, USA) working solution in MES (2-(*N*-morpholino)ethanesulfonic acid) buffer (Sigma-Aldrich, MO, USA). The mixtures were incubated over a temperature range 20–40°C at various pH from 2 to 9, and enzyme amount from 0.2 to 1.0 μg for 24 hr. In the inhibition assay, rGmcathB was pre-incubated with broad spectrum cysteine protease inhibitor E-64 at 10 μM for 30 min at 37°C before adding BODIPY FL casein. The fluorescence was detected in a fluorescence microplate reader (Fluoroscan Ascent, Thermo Fisher Scientific Inc., MA, USA) at 485 nm excitation and 518 nm emissions.

Hemoglobin and BSA degradation assay: Bovine hemoglobin (Hb) or bovine serum albumin (BSA) were mixed with rGmcathB that was pre-incubated with 10 mM L-cysteine for enzyme activation, and were treated with rGmcathB at the enzyme-substrate ratio 1 : 20 in 100 mM sodium acetate buffer (pH 4.0) with total reaction volume 600 μl. The reaction with Hb was incubated at 30°C for 0, 4, 18 and 24 hr, while the reaction with BSA was incubated for 0, 4, 48 and 72 hr. In the inhibition assay, reaction was done in duplicate using 10 μM of E-64. Results were separated by SDS-PAGE using non-reducing SDS loading buffer, and strained with Coomassie Brilliant Blue.

Determination of enzyme kinetic parameters: The hydrolysis efficiency of rGmcathB was analysed using fluorogenic peptidyl substrates which were purchased from Peptide Institute Inc, Osaka, Japan. The substrate was Z-Arg-Arg-MCA (benzyloxycarbonyl-L-arginyl-L-argine-4-methycoumaryl-7-amide) (Sigma-Aldrich, MO, USA) which is a selective substrate for cathepsin B. Z-Arg-Arg-MCA stock solution was prepared as 10 mM in dimethyl sulfoxide, and was diluted with deionized water to 1 mM before using. In order to activate rGmcathB, 1 μg of rGmcathB was preincubated with assay buffer (100 mM sodium acetate pH 4.0, 4 mM EDTA, 2.5 mM dithiothreitol) for 15 min at 30°C. Then the substrates were added in various final concentrations from 0.025 to 0.25 mM. Total

100 µl of assay were performed in 96-well plates in dark condition. The appearance of 7-amino-methylcoumarin was measured with 355 nm excitation and 460 nm emission by a fluorescence microplate reader (Fluoroscan Ascent, Thermo Fisher Scientific Inc., MA, USA). The hydrolysis of Hb was performed at concentration ranges from 25 to 500 µg/ml in sodium acetate buffer (pH 4.0). Hb solution (200 µl) was incubated with 3 µg of enzyme at 30°C for 60 minutes. The reaction was stopped with 250 µl of 10% trichloroacetic acid, centrifuged 15,000 rpm for 15 min, and the absorbance was read at 366 nm. All assays were repeated three times. K_M and K_{cat} values were obtained by fitting the initial rate to the Michaelis-Menten equation using nonlinear regression analysis (GraphPad Prism 5, MDF Co. Tokyo, Japan).

Results

Cloning and expression of cathepsin B

Computational analysis of GmcathB sequence predicted that the sequence encodes 310 of 340 amino acids of pro-cathepsin B (AF329480_1), including N-terminal signal peptide (13 amino acids, corresponding to amino acid position 18–30 in AF329480_1), pro-peptide (73 amino acids, corresponding to amino acid position 31–103 in AF329480_1), and active mature enzyme without C-terminal 13 amino acids (224 amino acids, corresponding to position 104–327 in AF329480_1) (Fig. 1). Inactive pro-enzyme has pro-peptide, which acts as an auto-inhibitor, and it needs to activate the enzyme by self-catalysis. The sequence has a conserved catalytic triad of cysteine protease (Cys116, His286 and Asn306 in AF329480_1) and possesses 13 conserved cysteine residues (Fig. 1). The analysis also indicated that Asn134 and Asn199 (in AF329480_1) were potential N-linked glycosylation sites. The amino acid sequence showed 78% identity to cathepsin B of flesh fly (*Sarcophaga peregrina*). GmcathB proenzyme (nucleotide position in full length GmcathB: 142 to 1,071) was cloned and expressed by both bacterial (*E. coli*) and yeast (*P. pastoris*) expression systems (Fig. 2). It has a calculated molecular mass of 35 kDa and PI 7.2. As expected, rGmcathB-GST fusion protein expressed by bacterial expression system was 61 kDa in its molecular mass, which is corresponds

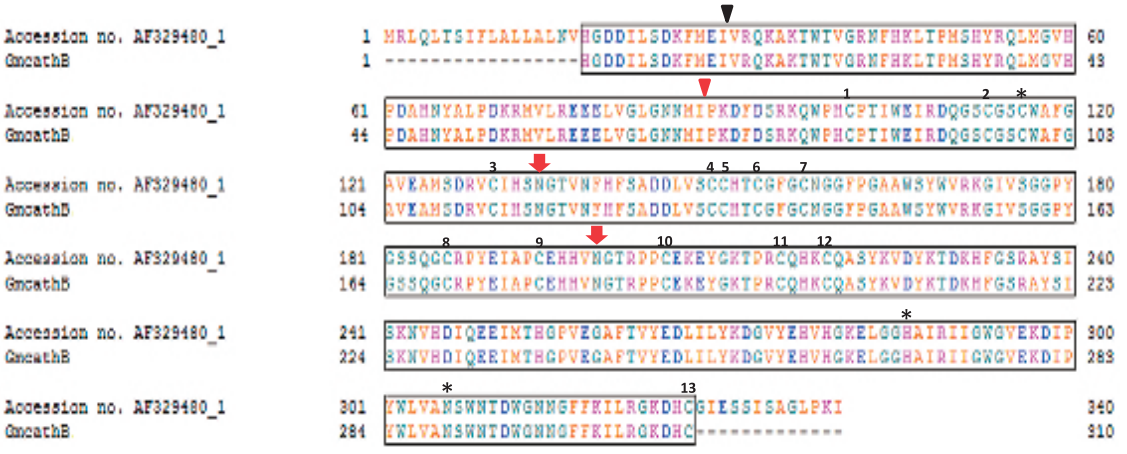

Fig. 1. Alignment of the deduced amino acid sequence of GmcathB from this study with *Glossina morsitans* cathepsin B (Accession No. AF329480_1). To confirm the sequence, the cloned GmcathB sequence was aligned with the sequence in the database (Accession No. AF329480_1). Cleavage site of signal peptide is shown by black arrow head. Start of the mature enzyme (amino acid residue from 89 to 340 in Accession No. AF329480_1) is shown by red arrow head. The sequence has catalytic triad of cysteine protease, Cys116, His286 and Asn306 (asterisks), 13 conserved cysteines (number 1 to 13), and two potential N-linked glycosylation sites, Asn134 and Asn 199 (red arrows).

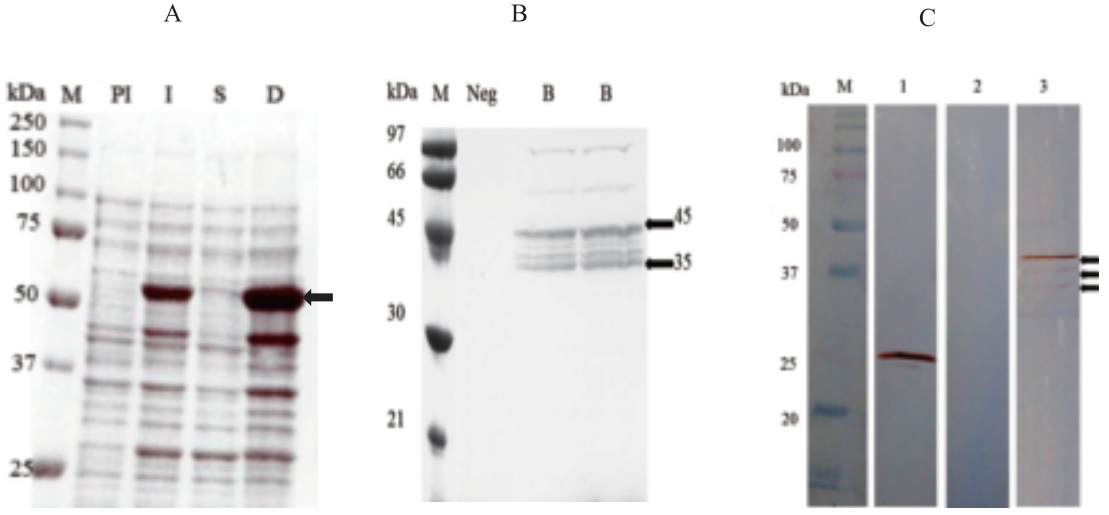

Fig. 2. Expression of the rGmcathB. Panel A: SDS-PAGE analysis of the rGmcathB in *E. coli* expression system, the GST-tagged-rGmcathB protein was expressed as 61 kDa protein (arrow). Total bacterial protein samples from before and after induction of the rGmcathB expression was electrophoresed at lanes PI and I, respectively. Soluble (S) and insoluble (D) fractions of the total protein was electrophoresed. Panel B: The rGmcathB was expressed as secreted soluble protein by using *Pichia pastoris* protein expression system, and electrophoresed culture supernatant from before (Neg) and after induction (B) under reducing condition. The upper band represented full length recombinant protein (45 kDa) and the lower band was an active rGmcathB (35 kDa). Panel C: Western blot analysis of the rGmcathB from *P. pastoris* with anti-GST-tagged-rGmcathB antibody. Lane 1: positive control (GST protein), Lane 2: negative control (*Pichia* stain 4 that contain pPinkα-HC vector). Lane 3: rGmcathB. The upper arrow indicates full length recombinant protein, middle and lower arrows show proenzyme and processed mature enzyme after activation with DTT, respectively. M indicate molecular size marker.

to sum of 35 kDa rGmcathB and 26 kDa GST (Fig. 2A). rGmcathB-GST from *E. coli* mostly expressed as insoluble form. On the other hand, rGmcathB from *P. pastoris* expression system was resulting at least two major soluble proteins of 35 and 45 kDa, which were recognized by anti-rGmcathB-GST mouse polyclonal antibody (Fig. 2B and 2C).

Proteolytic activity of recombinant GmcathB

The proteolytic activity of rGmcathB was only detected in the recombinant protein expressed by *P. pastoris* system. rGmcathB hydrolysed both synthetic dipeptidyl substrate (Z-Arg-Arg-MCA) and fluorogenic casein (BODIPY FL casein). Although rGmcathB showed broad pH range activity from pH 4.0 to 9.0, highest activity was observed at pH 4.0 for the two substrates (Fig. 3A). Dose dependency of activity was similar between rGmcathB and control enzyme (papain) (Fig. 3B). Although clear temperature preference of rGmcathB was observed at 30°C in fluorogenic casein (Fig. 3C), such temperature preference was not observed when synthetic dipeptidyl substrate (Z-Arg-Arg-MCA) was used (Fig. 3C). Activity was completely inhibited by the specific cysteine protease inhibitor E-64 (Fig. 3A and 3B).

Recombinant GmcathB hydrolysed bovine hemoglobin and bovine serum albumin

The ability of rGmcathB to hydrolyse Hb and BSA was performed at pH 4.0 and 30°C according to the result from proteolytic activity of fluorogenic casein (Fig. 3A and 3C). rGmcathB (5 μg) hydrolysed Hb and BSA (100 μg) after 18 hr and 48 hr of incubation, respectively. As the results, bands corresponding to Hb monomer (16 kDa) were weakly strained and BSA was digested into small fragments (Fig. 4A and 4B). Papain was employed as a positive control for enzymatic activity, and could not hydrolysed Hb and BSA at enzyme to substrate ratio 1 : 20. However increasing amount of papain (10 μg) hydrolysed both Hb and BSA at 18 hr and 4 hr of

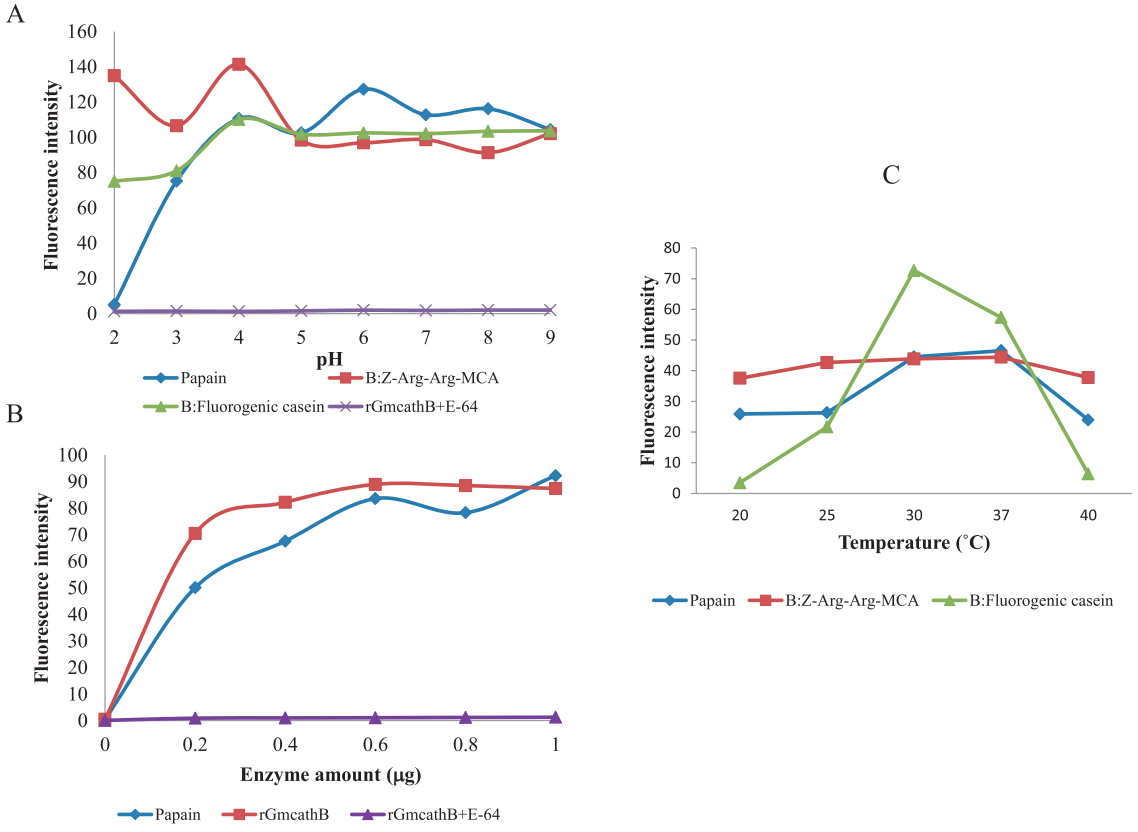

Fig. 3. Panel A: Effect of pH on proteolytic activity of the rGmcathB. Fluorogenic casein and Z-Arg-Arg-MCA are used as substrates. Papain and Z-Arg-Arg-MCA were used as a control proteolysis. The amounts of enzyme for fluorogenic casein and Z-Arg-Arg-MCA is 0.8 and 0.5 μg, respectively. E-64 is a specific cysteine protease inhibitor (E-64). Panel B: Dose dependency of rGmcathB and papain. Z-Arg-Arg-MCA was used as a substrate. Panel C: The effect of reaction temperature on the proteolytic activity at pH 4.0.

incubation, respectively (data not shown). The activity of rGmcathB on both substrates was totally inhibited by E-64.

The kinetic constants of GmcathB

rGmcathB was able to hydrolysed the substrates (Z-Arg-Arg-MCA), and the K_M values obtained from the substrates are 0.29 mM. (Table 2). Using bovine Hb as substrate, K_M value was 0.44×10^{-3} mM. The catalytic efficiency (K_{cat}/K_M) of rGmcathB for Z-Arg-Arg-MCA was 7.58 $mM^{-1}sec^{-1}$. On the other hand, hydrolytic activity toward Hb was 3.77×10^3 $mM^{-1}sec^{-1}$, which is approximately 7 times lower than that of papain (2.8×10^4) (Table 2).

Discussion

The previous studies in insect cathepsin B is proposed to play a crucial role in various physiological processes. In addition to the role in food digestion, metamorphosis and embryogenesis, previous studies reported that it took part in insect immune system which might kill the parasite[2,13]. Increasing interest on cathepsin B is focused in a wide range of pathogenic parasites causing malaria, Chagas disease, schistosomiasis and African trypanosomosis as well as their vectors[6,9,10,19,20,28]. The investigation on the role of systemic responses during trypanosome infection in tsetse suggested that digestive enzymes secreted in tsetse midgut play key roles not only in blood meal digestion but also in tsetse-trypanosome interaction[4,18]. It was reported that

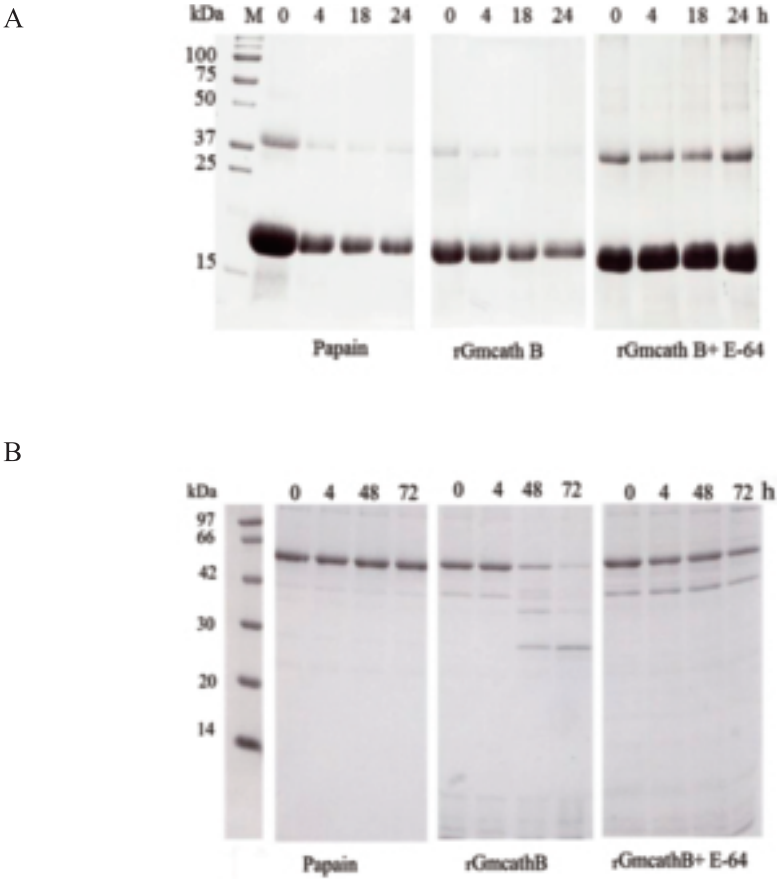

Fig. 4. The proteolytic activity toward bovine hemoglobin (Hb) and bovine serum albumin (BSA) were done at pH 4.0 at 30°C. Five μg of rGmcathB degraded Hb (100 μg) after 18 hr of incubation (Panel A) and BSA (100 μg) after 48 hr of incubation (Panel B). The activity was inhibited by cysteine protease inhibitor E-64.

Table 2. Enzyme assay of rGmcathB

Substrate (enzyme)	K_M (mM)	K_{cat} (sec^{-1})	K_{cat}/K_M (mM^{-1} sec^{-1})
Z-Arg-Arg-MCA (rGmcathB)	0.29	2.20	7.58
Bovine hemoglobin (rGmcathB)	0.44×10^{-3}	1.66	3.77×10^{3}
Bovine hemoglobin (Papain)	3.33×10^{-3}	94.03	2.8×10^{4}

midgut proteases cleaved the N-terminus of *T. brucei* procyclin and released the peptide that might have an influence on infection[1]. Although cathepsin B is one of tsetse midgut digestive enzymes, its enzyme characteristics have not been analyzed yet[28]. Gene cloning and subsequent sequence analyses revealed that the deduced amino acid sequence of GmcathB shares most of the common features to the previously reported tsetse cathepsin B[28]. GmcathB contained 13 cysteine residues and two possible N-linked glycosylation sites. These N-glycosylation sites may have roles in protein folding, structural stability, substrate recognition and secretion processes. The deduced amino acid sequence of GmcathB contained a catalytic triad (Cys116, His286 and Asn306), which was responsible for its proteolytic activity. rGmcathB expressed in prokaryotic expression system were insoluble form, and the renatured recombinant protein failed to show enzymatic activity (data not shown). In order to produce soluble and

enzymatically active rGmcathB protein, eukaryotic (*P. pastoris*) expression system was utilized because of many of the advantages of protein processing, protein folding, and the availability of posttranslational modifications. As a result, two major rGmcathB, 35 kDa and 45 kDa, was observed. Since the transcription of gene included the sequence of AOX1 priming site of pPinkα-HC vector, 85 amino acid residues with estimated molecular mass 10 kDa, the 45 kDa protein seemed to be the full length AOX1 fused rGmcathB. On the other hand, because of self-catalytic activity of the rGmcathB, 35 kDa protein might be mature enzyme which has proteolytic activity. This is supported by the study of cathepsin B-like protease from the cotton boll worm which reported that reducing condition using DTT plays a role in the activity of enzymes[34]. Although the highest proteolytic activity of rGmcathB was achieved at pH 4.0, its pH preference was not very strict (Fig. 3A). It was reported that tsetse midgut lumen was high pH[15]. This is probably one of the reasons why GmcathB shows catalytic activities in wide pH range. The optimal temperature of the enzyme was 30°C when fluorogenic casein was utilized as a substrate, and it seemed to be in range of the temperature of tsetse habitat. However, the optimal temperature was not very clear against Z-Arg-Arg-MCA substrate (Fig. 3C). In addition, K_{cat}/K_M was extremely low in Z-Arg-Arg-MCA (7.58) as compared to Hb (3.77×10^3) (Table 2). These results suggest that Z-Arg-Arg-MCA is not a suitable substrate for GmcathB. The hydrolysis activity of GmcathB (5 μg) toward Hb and BSA was observed after 18 and 48 h of incubation, respectively. These results indicated that the rGmcathB has higher catalytic activity to Hb than BSA (Fig. 4). In contrast, 10 μg of papain had higher catalytic activity to BSA than Hb (Fig. 4). These findings were also supported by the K_M values determined that GmcathB have higher affinity to Hb than papain (Table 2). In general, digestion processes consists of two steps corresponding to each midgut compartment, namely endo- and ecto-peritrophic membrane spaces. As a first step of digestion, dietary components, such as proteins, are cleaved in the endo-peritrophic membrane space (midgut lumen) by proteases to release small peptides. Then peptides are digested by peptidase located in the midgut epithelium and ecto-peritrophic membrane spaces[25]. Therefore further investigation is needed to clarify midgut localization of GmcathB. However, this is the first report to characterize the enzymatic activity of cathepsin B from tsetse fly.

Acknowledgements

This study was financially supported by a grant from the Graduate School of Obihiro University of Agriculture and Veterinary Medicine, the Global COE Program, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), and Japan and Japan Society for the Promotion of Science (JSPS).

References

1) Acosta-Serrano, A., Vassella, E., Liniger, M., Renggli, C. K., Brun, R., Roditi, I. and Englund, P. T. 2001. The surface coat of procyclic *Trypanosoma brucei*: Programmed expression and proteolytic cleavage of procyclin in the tsetse fly. *Proc. Natl. Acad. Sci. U. S. A.,* **98**: 1513–1518.
2) Ahn, J.-E., Guarino, L. A. and Zhu-Salzman, K. 2007. Seven-up facilitates insect counter-defense by suppressing cathepsin B expression. *Febs J.,* **274**: 2800–2814.
3) Atkinson, H. J., Babbitt, P. C. and Sajid, M. 2009. The global cysteine peptidase landscape in parasites. *Trends parasitol.,* **25**: 573–581.
4) Butikofer, P., Vassella, E., Boschung, M., Renggli, C. K., Brun, R., Pearson, T. W. and Roditi, I. 2002. Glycosylphosphatidylinositol-anchored surface molecules of *Trypanosoma congolense* insect forms are developmentally regulated in the tsetse fly. *Mol. Biochem. Parasitol.,* **119**: 7–16.

5) Carnevali, O., Cionna, C., Tosti, L., Lubzens, E. and Maradonna, F. 2006. Role of cathepsins in ovarian follicle growth and maturation. *Gen. Comp. Endocrinol.,* **146**: 195–203.
6) Cazzulo, J. J. 2002. Proteinases of *Trypanosoma cruzi*: Potential targets for the chemotherapy of chagas disease. *Curr. Top. Med. Chem.,* **2**: 1261–1271.
7) Cesen, M. H., Pegan, K., Spes, A. and Turk, B. 2012. Lysosomal pathways to cell death and their therapeutic applications. *Exp. Cell Res.,* **318**: 1245–1251.
8) Cho, W. L., Tsao, S. M., Hays, A. R., Walter, R., Chen, J. S., Snigirevskaya, E. S. and Raikhel, A. S. 1999. Mosquito cathepsin B-like protease involved in embryonic degradation of vitellin is produced as a latent extraovarian precursor. *J. Biol. Chem.,* **274**: 13311–13321.
9) Dvorak, J., Mashiyama, S. T., Braschi, S., Sajid, M., Knudsen, G. M., Hansell, E., Lim, K.-C., Hsieh, I., Bahgat, M., Mackenzie, B., Medzihradszky, K. F., Babbitt, P. C., Caffrey, C. R. and McKerrow, J. H. 2008. Differential use of protease families for invasion by schistosome cercariae. *Biochimie,* **90**: 345–358.
10) Hansen, G., Heitmann, A., Witt, T., Li, H., Jiang, H., Shen, X., Heussler, V. T., Rennenberg, A. and Hilgenfeld, R. 2011. Structural basis for the regulation of cysteine-protease activity by a new class of protease inhibitors in *Plasmodium*. *Structure,* **19**: 919–929.
11) Kageyama, T. and Takahashi, S. Y. 1990. Purification and characterization of a cysteine proteinase from silkworm eggs. *Eur. J. Biochem.,* **193**: 203–210.
12) Kaiser-Alexnat, R. 2009. Protease activities in the midgut of Western corn rootworm (*Diabrotica virgifera virgifera* LeConte). *J. Invertebr. Pathol.,* **100**: 169–174.
13) Koo, Y. D., Ahn, J. E., Salzman, R. A., Moon, J., Chi, Y. H., Yun, D. J., Lee, S. Y., Koiwa, H. and Zhu-Salzman, K. 2008. Functional expression of an insect cathepsin B-like counter-defence protein. *Insect. Mol. Biol.,* **17**: 235–245.
14) Lee, K. S., Kim, B. Y., Choo, Y. M., Yoon, H. J., Kang, P. D., Woo, S. D., Sohn, H. D., Roh, J. Y., Gui, Z. Z., Je, Y. H. and Jin, B. R., 2009. Expression profile of cathepsin B in the fat body of *Bombyx mori* during metamorphosis. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.,* **154**: 188–194.
15) Liniger, M., Acosta-Serrano, A., Van Den Abbeele, J., Renggli, C. K., Brun, R., Englund, P. T. and Roditi, I. 2003. Cleavage of trypanosome surface glycoproteins by alkaline trypsin-like enzyme(s) in the midgut of *Glossina morsitans*. *Int. J. Parasitol.,* **33**: 1319–1328.
16) Macleod, E. T., Darby, A. C., Maudlin, I. and Welburn, S. C., 2007. Factors affecting trypanosome maturation in tsetse flies. *PLoS One* **2**: e239.
17) Mort, J. S. and Buttle, D. J. 1997. Cathepsin B. *Int. J. Biochem. Cell Biol.,* **29**: 715–720.
18) Osir, E. O., Imbuga, M. O. and Onyango, P. 1993. Inhibition of *Glossina morsitans* midgut trypsin activity by D-glucosamine. *Parasitol. Res.,* **79**: 93–97.
19) Peacock, L., Cook, S., Ferris, V., Bailey, M. and Gibson, W. 2012. The life cycle of *Trypanosoma* (*Nannomonas*) *congolense* in the tsetse fly. *Parasit. Vectors.* **5**: 109.
20) Pearson, T. W. 2001. Procyclins, proteases and proteomics: dissecting trypanosomes in the tsetse fly. *Trends Microbiol.,* **9**: 299–301.
21) Ribolla, P. E. M., Daffre, S. and Debianchi, A. G. 1993. Cathepsin-B and acid phosphatase activities during *Musca domestica* embryogenesis. *Insect Biochem. Mol. Biol.,* **23**: 217–223.
22) Shiba, H. J., Uchida, D., Kobayashi, H. and Natori, M. 2001. Involvement of cathepsin B- and L-like proteinases in silk gland histolysis during metamorphosis of *Bombyx mori*. *Arch. Biochem. Biophys.,* **390**: 28–34.
23) Smooker, P. M., Jayaraj, R., Pike, R. N. and Spithill, T. W. 2010. Cathepsin B proteases of flukes: the key to facilitating parasite control? *Trends parasitol.,* **26**: 506–514.
24) Stephens, A., Rojo, L., Araujo-Bernal, S., Garcia-Carreno, F. and Muhlia-Almazan, A. 2012. Cathepsin B from the white shrimp *Litopenaeus vannamei*: cDNA sequence analysis, tissues-specific expression and biological activity. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.,* **161**: 32–40.
25) Terra, W. R. and Ferreira, C. 1994. Insect digestive enzymes: Properties, compartmentalization and function. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.,* **109**: 1–62.
26) Wang, X., Liu, B., Wang, G., Tang, B. and Xiang, J. 2008. Molecular cloning and functional analysis of cathepsin B in nutrient metabolism during larval development in *Meretrix meretrix*. *Aquaculture,* **282**: 41–46.
27) Xu, Y. S. and Kawasaki, H. 2001. Isolation

and expression of cathepsin B cDNA in hemocytes during metamorphosis of *Bombyx mori*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.,* **130**: 393–399.
28) Yan, J., Cheng, Q., Li, C. B. and Aksoy, S. 2002. Molecular characterization of three gut genes from *Glossina morsitans morsitans*: Cathepsin B, zinc-metalloprotease and zinc-carboxypeptidase. *Insect Mol. Biol.,* **11**: 57–65.
29) Yang, X.-M., Hou, L.-J., Wang, J.-X. and Zhao, X.-F. 2007. Expression and function of cathepsin B-like proteinase in larval hemocytes of *Helicoverpa armigera* during metamorphosis. *Arch. Insect Biochem. Physiol.,* **64**: 164–174.
30) Yang, X. M., Hou, L. J., Dong, D. J., Shao, H. L., Wang, J. X. and Zhao, X. F. 2006. Cathepsin B-like proteinase is involved in the decomposition of the adult fat body of *Helicoverpa armigera*. *Arch. Insect Biochem. Physiol.,* **62**: 1–10.
31) Yano, T., Takahashi, N., Kurata, S. and Natori, S. 1995. Regulation of the Expression of cathepsin-B in *Sarcophaga peregrina* (flesh fly) at the translational level during metamorphosis. *Eur. J. Biochem.,* **234**: 39–43.
32) Zavasnik-Bergant, T. and Turk, B. 2007. Cysteine proteases: destruction ability versus immunomodulation capacity in immune cells. *Biol. Chem.,* **388**: 1141–1149.
33) Zhao, X. F., Wang, J. X., Li, F. X., Sueda, S. and Kondo, H. 2005. Analysis of substrate specificity and endopeptidyl activities of the cathepsin B-like proteinase from *Helicoverpa armigera*. *Protein J.,* **24**: 219–225.
34) Zhao, X .F., Wang, J. X., Xu, X. L., Schmid, R. and Wieczorek, H. 2002. Molecular cloning and characterization of the cathepsin B-like proteinase from the cotton boll worm, *Helicoverpa armigera*. *Insect Mol. Biol.,* **11**: 567–575.