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ABSTRACT

Aspartic proteases are proposed as attractive drug targets of pathogens including apicomplexan parasites. In the present study, a gene encoding aspartic protease 3 of *Toxoplasma gondii* (TgASP3) was cloned, expressed and characterized. The gene fragment of putative functional domain of TgASP3 was expressed in *Escherichia coli* as a recombinant glutathione-S-transferase (GST) fusion protein (rTgASP3d). The native TgASP3 with molecular mass of 66-kDa from *T. gondii* tachyzoites was identified by Western blot analysis using anti-rTgASP3d mouse serum. In addition, the result from immunofluorescent antibody test (IFAT) using anti-rTgASP3d suggests that the native TgSAP3 is localized in the cytoplasm of *T. gondii* tachyzoites. On the other hand, the growth of *T. gondii* tachyzoites was significantly inhibited by an aspartic protease inhibitor-pepstatin A. These results suggest that the TgASP3 might be a novel therapeutic target for *T. gondii* infection.

Keywords: aspartic protease; TgASP3; *Toxoplasma gondii*

INTRODUCTION

Toxoplasma gondii is an apicomplexan parasite that infects most species of warm-blooded animals and humans. *T. gondii* infection can be fetal in immuno-compromised patients, and can cause severe birth defects if a pregnant woman develops a primary infection (Timothy and Soldati, 2004; Saeij *et al.*, 2005). Aspartic proteases are common enzymes in eukaryotes, highly conserved among apicomplexan protozoan parasites and become attractive drug targets (Conseil *et al.*, 1999; Coombs *et al.*, 2001; Klemba and Goldberg, 2005). It is known as plasmepsins in *Plasmodium falciparum* and have been believed to be excellent drug targets (Semenov *et al.*, 1998; Wyatt and Berry, 2002; Ersmark *et al.*, 2006; Baum *et al.*, 2008), but little is known about the role of aspartic proteases in *T. gondii*, closely related to malaria parasites. Seven putative aspartic proteases genes of *T. gondii* (TgASP1~TgASP7) were bioinformatically annotated (Shea *et al.*, 2007). Among these TgASPs, the TgASP1 has been studied well, and proposed as a drug target and vaccine candidate although it is not essential for parasite survival (Shea *et al.*, 2007; Polonais *et al.*, 2011; Zhao *et al.*, 2013). All other TgASPs are not characterized yet. The aim of this study is to characterize TgASP3 and to determine if this enzyme can be used as potential drug target for toxoplasmosis.

MATERIALS AND METHODS

Parasites and strains

T. gondii tachyzoites RH strain was used for gene amplification and protein localization, while PLK strain expressing the green fluorescent protein (GFP) was used for inhibition assay. The parasites were grown in Vero cells cultivated in Minimum essential medium Eagle (MEM) (Sigma, USA) containing 8% FBS (fetal bovine serum) and 1% penicillin streptomycin (Sigma, USA). Tachyzoites were purified as previously described (Masatani *et al*., 2013). Total RNA was extracted from *T. gondii* tachyzoites and cDNA was synthesized using Transcriptor First Strand cDNA Synthesis kit (Roche, USA).

Cloning of TgASP3 gene

The gene fragment containing functional domain of TgASP3d (AY592973) was amplified by PCR by using cDNA from *T. gondii* RH strain as template. Oligonucleotide primers with *Bam* HI and *Sal* I restriction enzyme sites (underline) were designed and used for amplification of the gene (DPF2, 5'-GCT GGATCC CAA TAC GTC GGA GTG ATT GGC-3'; DPR1, 5'- TTG GTC GGT ATC GCT CGC GCG GTCGAC ATC-3'). Amplification was performed in 50 μl of 1$\times$PCR buffer (New England Biolab, UK) containing of 1 unit *Taq* polymerase, 5 μl of cDNA template, 10 pmol of each primer, 2.0 mM concentration of $MgSO_4$ and 2.0 mM concentration of deoxynucleotide triphosphate. The PCR was performed as follow; denaturation at 96 ºC for 5 min followed by 30 cycles amplification of (96 ºC for 30 sec, 55 ºC for 1 min and 72 ºC for 1 min) then final extension at 72 ºC for 7 min. The purified PCR product was electrophoresed in 1.5% agarose gel (Genetic Nippon, Japan), stained with 3 μg/ml ethidium bromide (Roche, Germany) and purified using Qiagen PCR purification kit (Qiagen, USA). Purified product was cloned into multiple cloning site of pGEM-T-easy vector (Promega, USA). The obtained plasmid pGEM-T/TgASP3d was confirmed insertion by restriction enzymes and sequencing analysis using sequencer (Applied Biosystem, USA). The TgASP3 gene was sub-cloned into pGEX-4T-3 expression vector (GE Healthcare, UK). The resulted plasmid pGEX-4T-3/TgASP3d was checked for accurate insertion by restriction enzyme digestion and nucleotide sequencing.

Expression and purification of TgASP3 GST-fusion protein

The pGEX-4T-3/TgASP3d was transformed into *E. coli* (DH5α) cells, and the cells were cultured at 37 ºC until OD_{600nm} level reached 0.3-05 in LB medium supplemented with 20 μg/ml ampicillin. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and incubated at 23 ºC for 24 hours. The purification of rTgASP3d expressed in inclusion bodies of *E. coli* was carried out as previously described with some modifications (Hill *et al.*, 1994; Kimbita *et al.*, 2001).

Preparation of mouse anti rTgASP3 sera

Five 6-week-old female ICR mice (Clea, Japan) were intra-peritoneal immunized with 100 μg of the purified rTgASP3d protein emulsified with and equal volume of complete Freund's adjuvant (Difco, USA). Three additional boosters with 50 μg of rTgASP3d proteins with incomplete Freund's adjuvant (Difco) were intra-peritoneal administrated 2 weeks intervals. Mouse anti-rTgASP3d sera was collected at day 14 after last booster and stored at -30ºC before used. All mice used in this study were treated under the guiding principles for the care and use of research animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Japan.

Detection of native TgASP3 by Western blot analysis and IFAT

Western blot analysis was conducted in order to detect the native TgASP3 protein in *T. gondii* tachyzoites. Briefly, purified *T. gondii* 1×10^9 tachyzoites lysate was migrated in SDS-PAGE 12% polyacrylamide gel. Migrated proteins were transferred into immobilon-P transfer membrane (Millipore, USA), incubated with mice anti-rTgASP3d serum (1:100 dilution) and soaked in 1:1,000 dilution of horse radish peroxidase-conjugated goat anti mouse immunoglobulin G (Bethyl, USA). The membrane was reacted with 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2 to visualize the specific protein. On the other hand, localization of the native TgASP3 in *T. gondii* tachyzoites was confirmed by IFAT. Briefly, 1×10^6 tachyzoites/ml of *T. gondii* tachyzoites RH strain was infected into Vero cell monolayer on sterilized round micro cover glass (Matsunami, Japan) in the 12 well plates (Nunc, Denmark). The cover glass was washed with sterilized PBS and fixed in 4% paraformaldehyde for 15 min at day 2 post infection. The cover glass was incubated with anti-rTgASP3d mice sera (1:100 diluted with 3% fetal calve serum in PBS) then incubated with 1:500 times dilution of Alexa-Flour® 488 conjugated goat anti mouse immunoglobulin G (IgG) (Molecular Probes, USA). Parasite's nuclease was stained with 6.25 μg of propidium iodide (PI) (Molecular Probes) containing 50 μg/ml of RNase A (Qiagen, Germany) and mounted by 6 μl of 50% glycerol-PBS (vol/vol). Green fluorescent signals were observed under a confocal laser scanning microscope (TCS, NT Leica, Germany).

Inhibitory effect of pepstatin A on growth of *T. gondii* tachyzoites *in vitro*

Pepstatin A was dissolved in DMSO and prepared in different concentration at 500, 250, 100, 50, 25, 10, 5, 2.5, 1, 0.5 and 0.25 μM in MEM culture media (Invitrogen). Vero cell (1×10^5 cells/well) were grown on sterilized micro cover glass (Matsunami) in the 12 well plate for 24 hrs. *T. gondii* PLK strain 1×10^6 tachyzoites/well were then inoculated on the Vero cells as mentioned above and incubated for 4 hrs. Various concentrations of pepstatin A in MEM medium culture media were applied on Vero cell infected with *T. gondii* and incubated at 37ºC for 48 hrs in 5% CO_2 incubator. The culture parasites on micro cover glass were collected and fixed with 4% paraformaldehyde for 15 min, washed with sterilized distilled water then mounted by adding 6 μl of 50% glycerol-PBS (vol/vol) solution on glass slide. Parasitophorous vacuole and tachyzoite number per parasitophorous vacuole were evaluated under fluorescent microscope. Non-treated and 1% DMSO treated cultures were used as negative controls. The experiment was confirmed at least 3 times.

RESULTS AND DISCUSSION

In the first attempt, the full length of TgASP3 containing open reading frame (ORF) of 1,932 bp (GenBank accession no. AY592973) was amplified by PCR and then expressed as a recombinant GST fusion protein. However, we could not get the soluble form of recombinant protein successfully. It might be due to the high composition of hydrophobic amino acids of the protein. Thereafter, the fragment containing the putative enzymatic domain of TgASP3d (280-602 aa) was amplified, and expressed as a recombinant GST fusion protein (rTgASP3d). As a result, rTgASP3d was successfully purified as a soluble form. Phylogenetic analysis showed that TgASP3d was closely related to plasmepsins 9 and 10 of *P. falciparum* (Fig. 1A). Bioinformatics analysis showed that TgASP3d belong to pepsin retropepsin like superfamily, containing inhibitor binging sites, catalytic residues and active sites (Fig. 1B). Comparison of amino acid

sequence of TgASP3d (from RH strain) showed high similarity with that of ME49 strain from GenBank. However, histidine at 102 in ME49 strain was replaced by arginine in RH strain (Fig. 1C). An 876 bp fragment of TgASP3d gene was amplified by PCR (Fig. 2A). This fragment encodes 292 amino acids with predicted molecular mass of 32-kDa. It has 78%, 55%, 53% and 48% homologous to aspartic proteases from other apicomplexan parasites: *Eimeria tenella* (CDJ43222.1), *Babesia equi* (XP00482392.1), plasmepsin 9 (AAX22057.1) and plasmepsin 10 (AAW78308.1) of *P. falciparum*, respectively. It contains hydrophobic, hydrophilic and neutral residues as 51.08%, 25.39% and 23.53%, respectively. Recombinant TgASP3d was expressed as a GST-fusion protein with molecular mass of 58-kDa on SDS-PAGE (Fig. 2B). The rTgASP3d was mainly expressed in inclusion bodies of *E. coli*, and its insoluble form was dissolved and refolded using urea solution (Kimbita *et al.*, 2001). Mice anti-rTgASP3 reacted to the native protein localized on cytoplasm of *T. gondii* tachyzoites (Fig. 3A). On the other hand, a 66-kDa of native TgASP3 was detected in *T. gondii* tachyzoites lysates (Fig. 3B). These results suggest that TgASP3 highly expressed in the tachyzoites stage and might be important to survival of parasite as previously described (Shea *et al.*, 2007).

Pepstatin A, an aspartic protease inhibitor significantly reduced the number parasitophorous vacuole of *T. gondii in vitro* from 0.5 μM and killed all parasites from 50 μM (Fig. 4A). Observation of tachyzoites in the parasitophorous vacuole showed that number of tachyzoites in parasitophorous vacuole was reduced in dose dependent manner (Fig. 4B). Moreover, pepstatin A treated parasite shown morphological changed as swollen and death (Fig. 4C). Further study is needed to link between parasite death and inhibition of the TgASP3.

In conclusion, this study provides primary information on the expression and localization of TgASP3 and suggests that this molecule might be a novel drug target for treatment of toxoplasmosis.

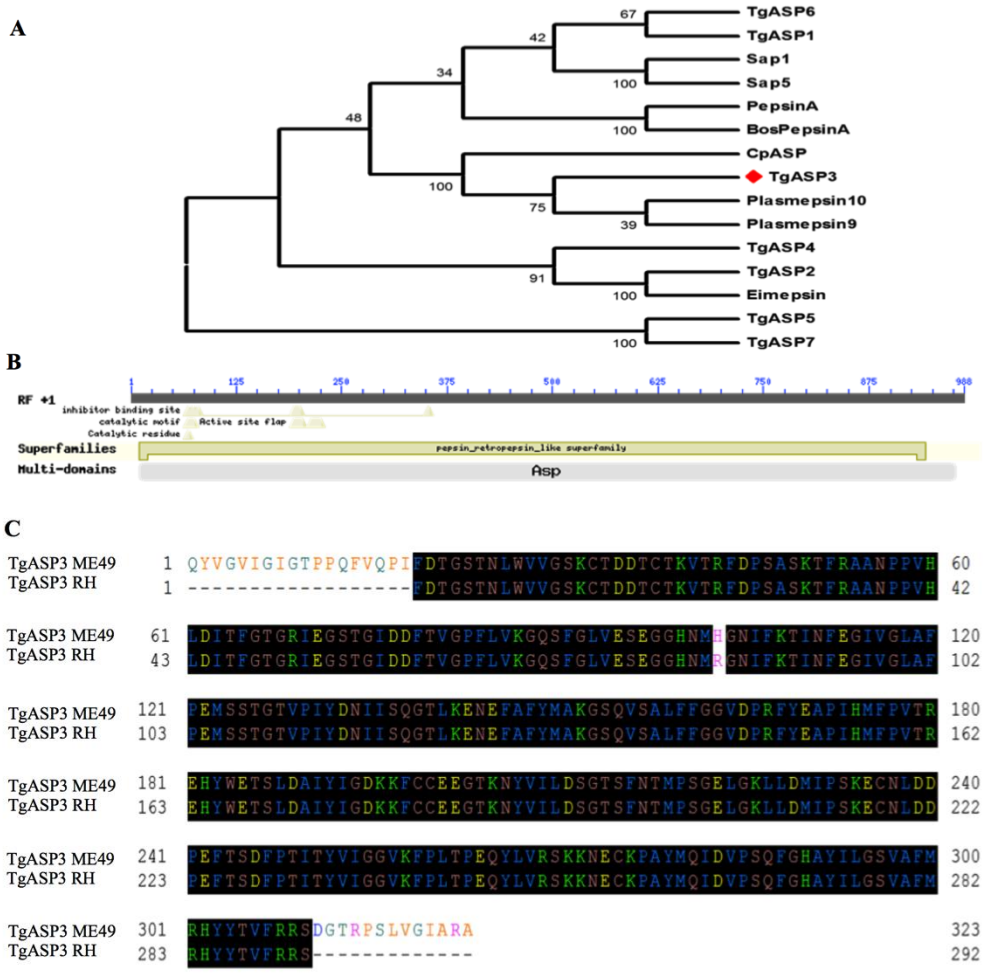

Fig. 1. Bioinformatics analysis of TgASP3 amino acid sequence. (A) Phylogenetic analysis of TgASP3 with aspartic proteases from other apicomplexan protozoan parasites using neighbor joining method in cooperated with Mega software 3.1. (B) Prediction of TgASP3 domains using BLAST. (C) Comparison of putative enzymatic domain of TgASP3 of ME49 strain (AY592973) and RH strain from this study using Genetyx ver. 7.

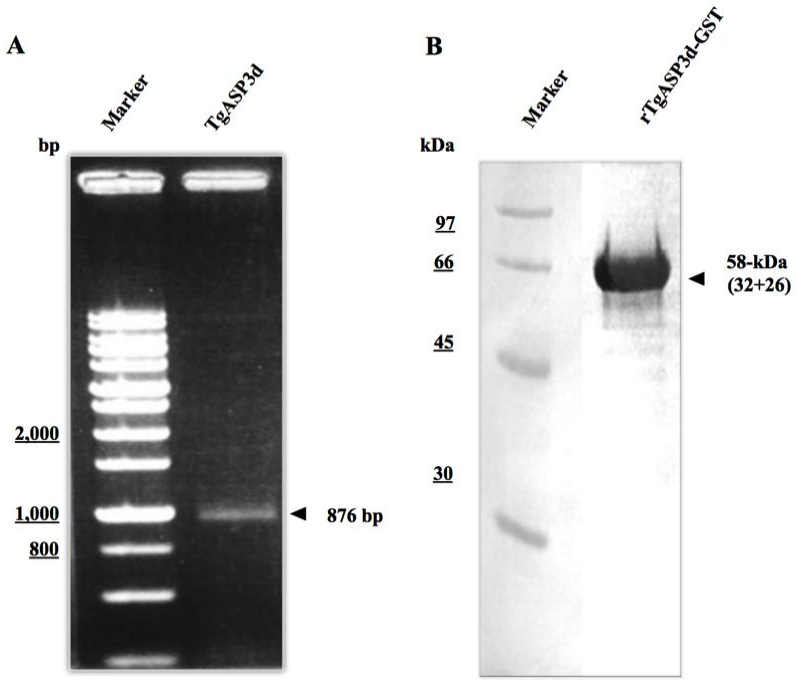

Fig. 2. Amplification and expression of TgASP3. (A) Gel electrophoresis showed the PCR product of the fragment of TgASP3 gene. (B) SDS-PAGE analysis showed purified rTgASP3-GST fusion protein.

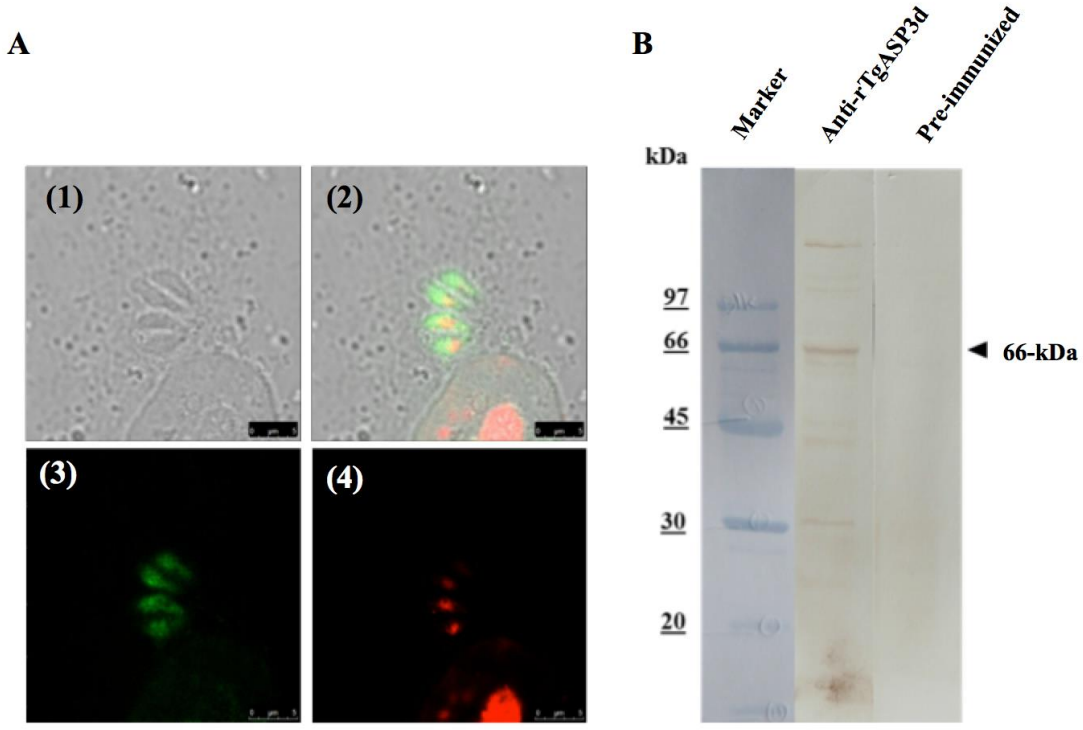

Fig. 3. Detection of native TgASP3. (A) Localization of TgASP3 on *T. gondii* tachyzoites: (1) phase contrast, (2) merge, (3) anti-rTgASP3 mice sera, and (4) DPI staining. (B) Western blot analysis showed the reaction of mice anti-rTgASP3 with *T. gondii* tachyzoite lysate.

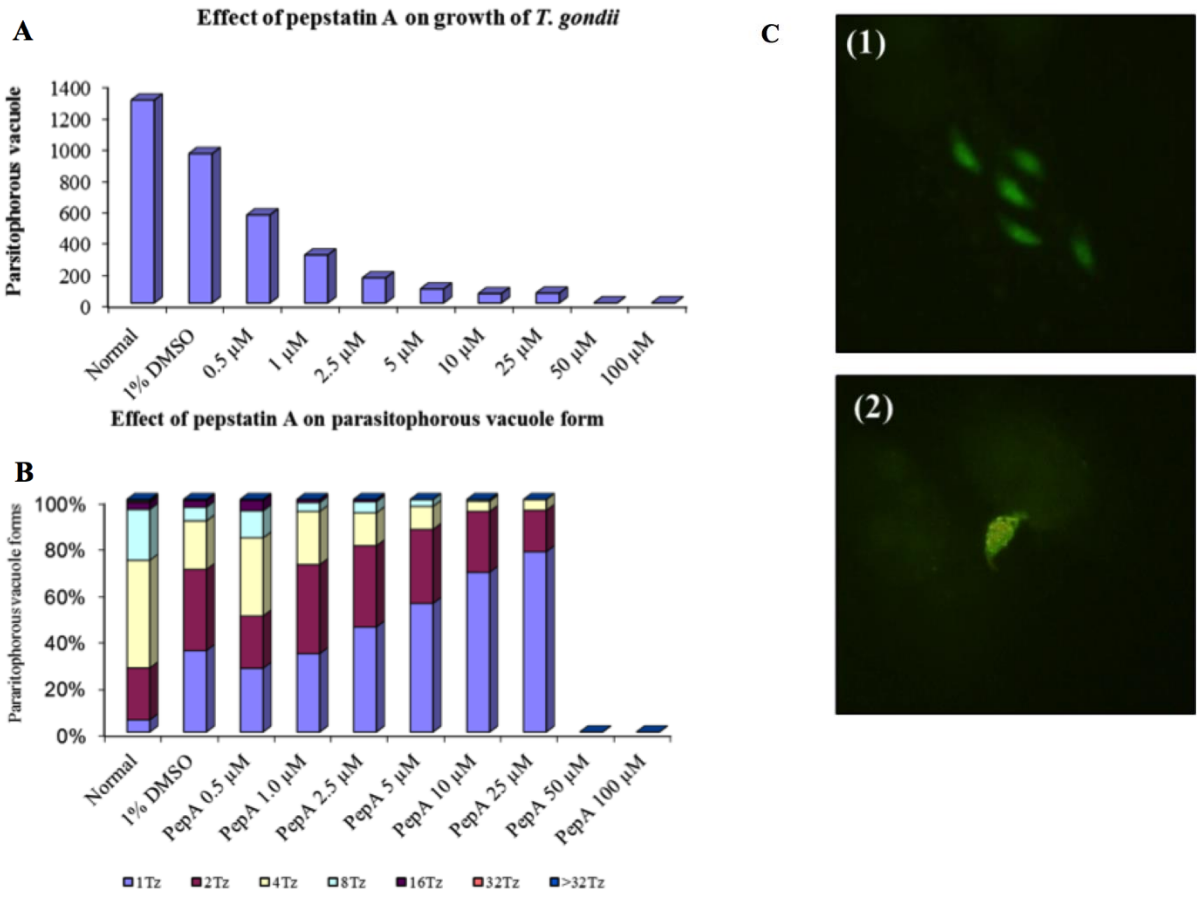

Fig. 4. Inhibitory effects of pepstatin A on the growth of *T. gondii in vitro*. (A) Effect of pepstatin A on the number of parasitophorous vacuole. (B) Effect of pepstatin A on the number of tachyzoite in the parasitophorous vacuole (parasitiphorous vacuole form). (C) Morphological change of *T. gondii* tachyzoites: (1) non treated parasites, (2) treated parasite.

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