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Echinococcus multilocularis: Identification and functional characterization of cathepsin B-like peptidases from metacestode ☆

Yasuhito Sako [a,*], Kazuhiro Nakaya [b], Akira Ito [a]

[a] Department of Parasitology, Asahikawa Medical University, Midorigaoka Higashi 2-1, Asahikawa, 078-8510 Hokkaido, Japan
[b] Animal Laboratory for Medical Research, Asahikawa Medical University, Midorigaoka Higashi 2-1, Asahikawa, 078-8510 Hokkaido, Japan

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ABSTRACT

Cysteine peptidases have potent activities in the pathogenesis of various parasitic infections, and are considered as targets for chemotherapy and antigens for vaccine. In this study, two cathepsin B-like cysteine peptidases (EmCBP1 and EmCBP2) from *Echinococcus multilocularis* metacestodes were identified and characterized. Immunoblot analyses demonstrated that EmCBP1 and EmCBP2 were present in excretory/secretory products and extracts of *E. multilocularis* metacestodes. By immunohistochemistry, EmCBP1 and EmCBP2 were shown to localize to the germinal layer, the brood capsule and the protoscolex. Recombinant EmCBP1 and EmCBP2 expressed in *Pichia pastoris*, at optimum pH 5.5, exhibited substrate preferences for Z-Phe-Arg-MCA, Z-Val-Val-Arg-MCA, and Z-Leu-Arg-MCA, and low levels of hydrolysis of Z-Arg-Arg-MCA. Furthermore, recombinant enzymes degraded IgG, albumin, type I and IV collagens, and fibronectin. These results suggested that EmCBP1 and EmCBP2 may play key roles in protein digestion for parasites' nutrition and in parasite–host interactions.

1. Introduction

Alveolar echinococcosis (AE), caused by the larval stage of *Echinococcus multilocularis*, is a serious parasitic disease of humans in countries of the higher latitudes of Northern Hemisphere. In the previous decade, a lot of new data have been published on prevalence of *E. multilocularis* in final and intermediate hosts in areas where it had previously not been recorded (Eckert et al., 2000; Ito et al., 2010). Humans are accidentally infected with *E. multilocularis* by ingestion of eggs excreted with the feces of carnivores harboring adult tapeworm of this species. It is thought that humans become exposed to *E. multilocularis* by handling of infected definitive hosts, or by ingestion of food and water contaminated with eggs. Oncospheres hatched from eggs in the small intestine of humans migrate via the portal system into various organs, mainly liver and differentiate and develop into the metacestode stage. The metacestodes propagate asexually like a tumor leading to organ dysfunction. Since clinical symptoms usually do not become evident until 10 or more years after initial parasite infection, early diagnosis and treatment especially during asymptomatic period are important for reduction of morbidity and mortality (McManus et al., 2003). About one third of patients have cholestatic jaundice and about one third of patients have epigastric pain. In the remaining patients, *E. multilocularis* infections are incidentally detected during medical examination for symptoms such as fatigue, weight loss, hepatomegaly (Pawlowski et al., 2001). In addition to surgical removal of alveolar hydatid cyst, treatment with antiparasitic agents, benzimidazole derivatives, is the most important for AE therapy. However, these drugs have parasitostatic activity rather than parasitocidal activity, and side-effects such as liver damage are often observed with long-term administration (Kern, 2006). Thus, it is urgent to develop novel reliable chemotherapeutic agents.

Cysteine peptidases belonging to clan CA family C1 (Rawlings et al., 2004; http://merops.sanger.ac.uk/) besides their housekeeping functions such as protein turnover in parasite cells are involved in evasion from host immune responses, essential nutrient uptake, and tissue penetration, by degrading host proteins, including immunoglobulin, complement components, kininogen, haemoglobin, albumin, and extracellular matrix proteins (reviewed by Sajid and McKerrow 2002; Dalton et al., 2003; Caffrey et al., 2004; Rosenthal, 2004; McKerrow et al., 2006; Robinson et al., 2008; Smooker et al., 2010). Furthermore, some cysteine peptidases have activities to stimulate human eosinophils to induce degranulation (Shin et al., 2005), deplete CD4 positive human lymphocytes *in vitro* (Molinari et al., 2000), induce apoptosis in human CD4 positive cells (Tato et al., 2004), by interacting with host cells via unknown mechanisms. Therefore, cysteine peptidases of parasites are considered as important targets for chemotherapy and/or

☆ *Note:* The nucleotide sequence data reported in this study are available in the GenBank, EMBL, and DDBJ databases under accession numbers AB586072 (EmCBP1) and AB586073 (EmCBP2).

* Corresponding author. Fax: +81 166 68 2429.
E-mail address: yasusako@asahikawa-med.ac.jp (Y. Sako).

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immunoprophylaxis (Dalton et al., 2003; Barr et al., 2005; Abdulla et al., 2007; Alcala-Canto et al., 2007).

E. multilocularis metacestodes survive for many years in human host, which leads us to consider peptidases as important parasite components to evade from host immune responses and to uptake nutrient. However, precise characterization of *E. multilocularis* peptidases essential to develop enzyme inhibitors has been hampered by the difficulty of obtaining pure parasite materials, since parasite materials obtained from laboratory animals are contaminated with numerous host cells resulting from that *E. multilocularis* infiltrate and proliferate by exogenous budding of the germinative cells in host tissue (Thompson, 1995). Recently, we have succeeded in cloning of cathepsin L-like peptidase genes (EmCLP1 and EmCLP2) from *E. multilocularis* metacestodes, which enabled us to prepare a large amount of parasite enzymes for detailed characterizations (Sako et al., 2007). Activities of recombinant EmCLP1 and EmCLP2 to degrade human IgG, bovine albumin, type I and type IV collagen and fibronectin have been demonstrated, which suggests their important roles in parasite growth and survival in the host. In the present study, we have identified two genes encoding cathepsin B-like cysteine peptidases from *E. multilocularis* metacestodes. Immunoblot analyses with monoclonal antibody detected both enzymes in crude metacestode extract and ES products, and immunohistochemical studies revealed that both enzymes are expressed in the germinal layer, the brood capsule, and the protoscolex. Moreover, enzymatic activities against synthetic peptide substrates and macromolecule proteins were also characterized by using recombinant active enzymes expressed in *Pichia pastoris*.

2. Materials and methods

2.1. Animals

Animal procedures and management protocols in this study were approved by the Ethics Committee of Asahikawa Medical University, Asahikawa, Japan.

2.2. Preparation of parasite material

E. multilocularis (Furano isolate, Hokkaido, Japan) metacestode tissue was obtained from non-obese diabetic severe combined immunodeficiency (NOD/Shi-*scid*) mice infected by intraperitoneal passage of metacestodes (Nakaya et al., 2006). Microvesicle and protoscolex suspensions were prepared by pressing metacestode tissue through a 300 μm metal mesh with PBS. The microvesicles and protoscoleces were washed five to seven times with PBS, and then used for preparation of metacestode crude lysate and excretory/secretory (ES) products. Because NOD/Shi-*scid* mice had little inflammatory response, isolation of microvesicles and protoscoleces with less host components, which are commonly found in those from immunocompetent mice, could be done efficiently.

To prepare metacestode crude lysate, microvesicles and protoscoleces were homogenized with three times volume of lysis buffer consisting of 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1.0% 3-[(3-cholamidopropyl)dimethylammonia]-1-propanesulfonic acid (CHAPS) in the presence of peptidase inhibitors (protease inhibitor cocktail for mammalian tissues, Sigma–Aldrich). After one freeze–thaw cycle and centrifugation at 10,000*g* for 30 min at 4 °C, the supernatant was recovered and kept at −80 °C until use.

To obtain ES products, microvesicles and protoscoleces were cultured in RPMI-1640 medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C for 12 h. Few dead microvesicles and protoscoleces were found under a microscopic examination at the end of cultivation, which indicated that the contamination of intracellular proteins released into the medium supernatant as a result of parasite death had been almost completely avoided. The medium supernatant containing ES products was carefully collected and was passed through a disposable chromatography column (Econo-Pac column, Bio-Rad) with a porous bed support (a 30 μm pore size) to remove minor microvesicle and protoscolex contaminants. After filtration through 0.45 μm filter membrane (Millipore), the medium supernatant was concentrated by using an Amicon Ultra-15 Centrifugal Filter Unit with a cutoff size of 5 kDa (Millipore) and kept at −80 °C until use.

2.3. Cloning of EmCBP1 and EmCBP2 genes

Total RNA was isolated from freshly prepared *E. multilocularis* metacestodes using Trizol reagent (Gibco BRL) according to the manufacture's instruction. After purification of Poly(A)$^+$ RNA by using oligo(dT)-latex beads (TaKaRa), cDNA available in 5′ and 3′ rapid amplification of cDNA end (RACE) method was synthesized from 1 μg of purified poly(A) $^+$ RNA by using the GeneRacer Kit (Invitrogen).

3′ RACE were performed with degenerated forward primers designed from the consensus sequences flanking the active site residues of eukaryotic cysteine peptidases and 3′ RACE primer.

The forward primer (5′-CAGGGTCAGTGYGGNTCNTGYTGG-3′) and GeneRacer 3′ primer (5′-GCTGTCAACGATACGCTACGTAACG-3′) were used in the first round PCR, and the forward primer (5′-CAGTGCGGTTCNTGYTGGGCNTTY-3′) and GeneRacer 3′ Nested primer (5′-CGCTACGTAACGGCATGACAGTG-3′) were used in the nested PCR. PCR reactions were performed in a 50 μl of reaction mixture containing 1 × *Ex Taq* Buffer, 2.0 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM of each dNTP, 5 ng of cDNA and 0.5 units of *EX Taq* DNA polymerase (TaKaRa) and cycling conditions were 30 s at 94 °C (first cycle: 2 min at 94 °C), 30 s at 50 °C and 30 s at 72 °C for 30 cycles. The PCR products were separated in a 1.0% agarose gel, the DNA fragments were recovered and cloned into pGEM T-vector (Promega), and plasmid clones were sequenced. To obtain sequence upstream of EmCBP1 and EmCBP2 genes, 5′ RACE was performed using gene-specific primer (5′-CGTACCATCACTGCTC TCCCGCTTACTGTC-3′ for EmCBP1gene, 5′-TGCAACCAAAGCCACAG AATAAGCC-3′ for EmCBP2 gene) and GeneRacer 5′ primer (5′-CG ACTGGAGCACGAGGACACTGA-3′) with annealing temperature of 60 °C. Finally, full-length cDNAs of the EmCBP1 and EmCBP2 genes were cloned by PCR using a high-fidelity DNA polymerase, Phusion DNA polymerase (Finnzymes), and primers directed to both the UTR ends.

2.4. Expression of the mature region of EmCBP1 and EmCBP2 in Escherichia coli and purification

The mature enzyme region of EmCBP1 or EmCBP2 was amplified by PCR with primer sets containing a restriction enzyme (underlined) recognition sequence added to 5′ end to facilitate cloning of the PCR products. The primers used were: 5′-GGGAATTC CTGCCGGCATCTTTTGATCCC-3′ (mCBP1/F), 5′-GGGTCGACTAGTT TTGTGGGATACCTGC-3′ (CBP1/R), 5′-GGGAATTCCTTCCCTCAGAAT TTGACGCA-3′ (mCBP2/F), 5′-GGGTCGACTCACTTCCTTATTTTTGGA ATACC-3′ (CBP2/R). The PCR reactions were performed with cDNA clones as templates. The PCR products were digested with *Eco*RI and *Sal*I, cloned into bacterial expression vector pET-30a(+) (Novagen) for producing a fusion protein with His tag. The cloned plasmids were transfected into *E. coli* BL21(DE3)pLysS strain. Expression of recombinant proteins was induced by addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) to the culture. Recombinant proteins were purified using Ni-NTA beads (Qiagen) under denaturation conditions. Protein concentration was determined by BCA protein assay kit (Pierce).

2.5. Production of monoclonal antibodies

Female BALB/c mice were immunized by intraperitoneally (i.p.) injection of 50 μg of *E. coli*-expressed recombinant EmCBP1 (eEmCBP1) or EmCBP2 (eEmCBP2) emulsified in Freund's complete adjuvant. Three weeks later the procedure was repeated but with Freund's incomplete adjuvant. Three days before the fusion, the mice were i.p. boosted with 50 μg antigens in PBS. Spleen cells of mice were fused with NS-1 myeloma cells. The antibody-secreting hybridomas were screened by ELISA with eEmCBP1 or eEmCBP2. Hybridomas selected were cloned by limit dilution at least twice.

2.6. SDS–PAGE and immunoblot analysis

Proteins were treated with a SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2.0% SDS, 50 mM dithiothreitol and 10.0% glycerol) at 100 °C for 5 min and separated in a 7.5% or 12.5% polyacrylamide gel. For immunoblot analysis, the separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane sheet (Millipore). The sheet was blocked with blocking solution (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1.0% casein, 0.1% Tween 20) and probed with monoclonal antibody followed by alkaline phosphatase-conjugated anti-mouse IgG antibody (Novagen). Nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate was used for color development.

2.7. Immunohistochemistry

Parasite tissues and livers from infected-NOD/Shi-*scid* mice were washed once with PBS and fixed with 2.0% paraformaldehyde in PBS overnight at 4 °C and then embedded in paraffin. Sections of 5 μm were produced and were transferred to slides. After antigen retrieval using HistoVT One (Nacalai Tesque), sections were treated with peroxidase blocking solution (0.3% H_2O_2 in methanol) for 30 min. Then, sections were washed with PBS, blocked using blocking solution for 1 h and incubated overnight at 4 °C with monoclonal antibody. After three washing with PBS, the sections were incubated with peroxidase-conjugated anti-mouse IgG antibody (ImmPRESS REAGENT, Vector laboratories) for 30 min at room temperature. After four washing with PBS, the sections were incubated with 3-amino-9-ethylcarbazole. All sections were counterstained with hematoxylin.

2.8. Expression of EmCBP1 and EmCBP2 in P. pastoris

The pro-mature coding region of EmCBP1 or EmCBP2 was amplified by PCR with primer sets containing a restriction enzyme (underlined) recognition sequence. The primers used were: 5′-CGGAATTCA GTACTGTGACCAGCGCAATTGG-3′ (proCLP1/F), 5′-ATGCGGCCGCTA GTTTTGTGGGATACCTGC-3′ (PIC CLP1/R), 5′-CGGAATTCAGAAAACC TCATCAGAGCGAC-3′ (proCLP2/F), 5′-ATGCGGCCGCTCACTTCCTTA TTTTTGGAATACC-3′ (PIC CBP2/R). The PCR reactions were carried out as mentioned above. The PCR products were digested with *Eco*RI and *Not*I and cloned into yeast expression vector pPICZα A (Invitrogen), and subsequently linearized with *Pme*I and electroporated into *P. pastoris* KM71 host cells. Yeast transformants were cultured in 500 ml of buffered-glycerol complex medium (1.0% yeast extract, 2.0% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1.0% glycerol, and 100 mM potassium phosphate, pH 6.0) at 28 °C for 2 days and collected by centrifugation at 1000*g* for 5 min, and protein expressions were induced by resuspending the cells in 100 ml of buffered-methanol minimal medium (1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1.0% methanol, and 100 mM potassium phosphate, pH 6.0) at 28 °C for 3 days. Due to the presence of an α-factor leader peptide sequence, recombinant proteins were secreted into expression medium. The culture medium containing recombinant EmCBP1 (yEmCBP1) or EmCBP2 (yEmCBP2) was collected, concentrated using an Amicon stirred cell with a YM-10 membrane and dialyzed against 50 mM sodium acetate buffer (pH 4.5) containing 2.5 mM EDTA. For purification of active form of yEmCBP1, dialysate was directly loaded on a HisTrap SP XL cation-exchange column pre-equilibrated with 50 mM sodium acetate buffer (pH 4.5) containing 2.5 mM EDTA after activation at 37 °C for 1 h in the presence of 10 mM L-cysteine and proteins were eluted by use of a linear salt gradient (0–1.0 M NaCl). For purification of active form of yEmCBP2, the conversion of pro-form into active enzyme was accomplished by treatment with pepsin. After addition of porcine pepsin (Sigma–Aldrich) at a final concentration of 0.5 mg/ml, the activation mixture was incubated at 37 °C for 4 h. The activated yEmCBP2 was purified as mentioned above. Recombinant proteins were treated with peptide:*N*-glycosidase F (PNGase F, New England Biolabs) under denaturing conditions to remove any N-linked oligosaccharides to determine whether recombinant proteins were glycosylated.

2.9. Irreversible active site-labelling of yEmCBP1 and yEmCBP2

A biotinylated dipeptidyl fluoromethylketone (Biotin-Phe-Ala-FMK, MP Biomedicals), a cysteine peptidase inhibitor, was used for active-site labelling. Briefly, the purified enzyme was incubated for 30 min at room temperature with 10 μM biotin-Phe-Ala-FMK in 100 mM sodium acetate buffer (pH 5.5) containing 2.5 mM EDTA, 0.1% CHAPS and 10 mM L-cystine. Labelled proteins were detected with alkaline phosphatase-conjugated streptavidin (Novagen).

2.10. Substrate specificity and kinetic measurements of yEmCBP1 and yEmCBP2

Peptidase activity was characterized by using peptidyl-4-methylcoumarin-7-amide (MCA) as substrates. The standard assay volume was 200 μl, using 100 mM sodium acetate buffer (pH 5.5) containing 0.1% CHAPS, 2.5 mM EDTA and 10 mM L-cysteine. Substrates were added to a final concentration of 2 μM, or other concentration as required. Assays were performed at room temperature. The amount of 7-amino-4-methylcoumarin (AMC) released was measured by the fluorometer (VersaFluor Fluorometer, Bio-Rad) at an excitation wavelength of 370 nm and an emission wavelength of 460 nm.

For determination of the optimum pH of recombinant enzyme activity, 100 mM citrate–phosphate buffer (pH 3.0–8.0) containing 250 mM NaCl, 0.1% CHAPS, 2.5 mM EDTA and 10 mM L-cystine, were used. Substrate specificities were investigated using benzyloxycarbonyl (Z)-Phe-Arg-MCA, Z-Arg-Arg-MCA, Z-Leu-Arg-MCA, Z-Gly-Pro-Arg-MCA, and Z-Val-Val-Arg-MCA at a concentration of 2 μM. The values of K_m and V_{max} for Z-Phe-Arg-MCA and Z-Arg-Arg-MCA were determined by a nonlinear regression analysis. The molar concentration of active recombinant enzymes was determined by active-site titration using the Z-Phe-Arg-MCA and cysteine peptidase inhibitor *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) as described by Barrett and Kirschke (1981). All peptidyl-MCA substrates used were purchased from Peptide Institute, Japan.

2.11. Inactivation kinetics of yEmCTB1 and yEmCTB2

Inactivation of recombinant enzyme was performed in 100 mM citrate–phosphate buffer (pH 6.5, 7.0, and 7.5) containing 250 mM NaCl, 0.1% CHAPS, 2.5 mM EDTA, and 10 mM L-cystine in the presence of 50 μM Z-Phe-Arg-MCA. Progress of the reaction was monitored continuously by the fluorescence of the released products for 30 min. All progress curves obtained were exponential, and could be best fitted to the following first-order relationship (Eq. 1):

$$P - P_{\infty}(1 - e^{k_{obs}t})$$

where P and P_{∞} are the product concentration at a given or infinite time, respectively, and k_{obs} is the observed first-order inactivation rate constant. And half-lives were calculated as $t_{1/2} = \ln 2/k_{obs}$.

2.12. Protein substrate digestion

Human IgG, human serum albumin (HSA), soluble calf skin type I collagen, human placenta type IV collagen, and bovine plasma fibronectin were used as protein substrates. All protein substrates used were purchased from Sigma–Aldrich, Japan. Active recombinant enzyme (0.2 μM) was incubated with 0.2 mg/ml of each protein substrate in 100 mM sodium acetate buffer (pH 5.5) containing 2.5 mM EDTA and 10 mM L-cysteine at 37 °C for 4 h. The digestion reaction was stopped by the adding of SDS–PAGE sample buffer. Protein substrate digests were subjected to SDS–PAGE and degradation products were visualized by Coomassie Blue staining.

3. Results

3.1. Primary structure of EmCBP1 and EmCBP2

Following PCR with degenerate primers and sequencing analysis of 48 PCR product clones, a total of four different partial genes encoding cysteine peptidases were obtained from *E. multilocularis* metacestode cDNA. Database search of the deduced amino acid sequences of individual clones revealed that two clones are identical to EmCLP1 and EmCL2 (Sako et al., 2007), respectively, and other two clones show high homology to cathepsin B-like peptidases. Rapid amplification of cDNA ends (RACE) was performed to obtain the full-length of two novel cysteine peptidase cDNAs, termed EmCBP1 and EmCBP2, respectively.

As shown in Fig. 1, EmCBP1 consists of a 18-residue putative signal sequence predicted by the method of Nielsen et al. (1997), a 77-residue propeptide and the 256-residue mature enzyme. EmCBP2 consists of a 16-residue putative signal sequence, a 67-residue propeptide and the 255-residue mature enzyme. Comparison of EmCBP1 with EmCBP2 revealed an amino acid identity of 60.5% (65.6% for the mature region only). The catalytic triad residues are conserved with other crucial residues shaping an oxyanion hole (Menard et al., 1991), and the occluding loop region (Musil et al., 1991) responsible for peptidyldipeptidase activity, a unique feature of cathepsin B, is also present in the mature enzyme. The predicted molecular masses of the mature EmCBP1 and EmCBP2 are 28,241 and 28,140 Da, respectively. In mature region, EmCBP1 has one putative N-linked glycosylation site at position 116.

3.2. Detection of EmCBP1 and EmCBP2 in E. multilocularis

Immunoblot analyses of *E. multilocularis* metacestode crude lysate (Fig. 2, lanes 1) and ES products (lanes 2) were performed. Anti-EmCBP1 monoclonal antibody recognized proteins of 24.5

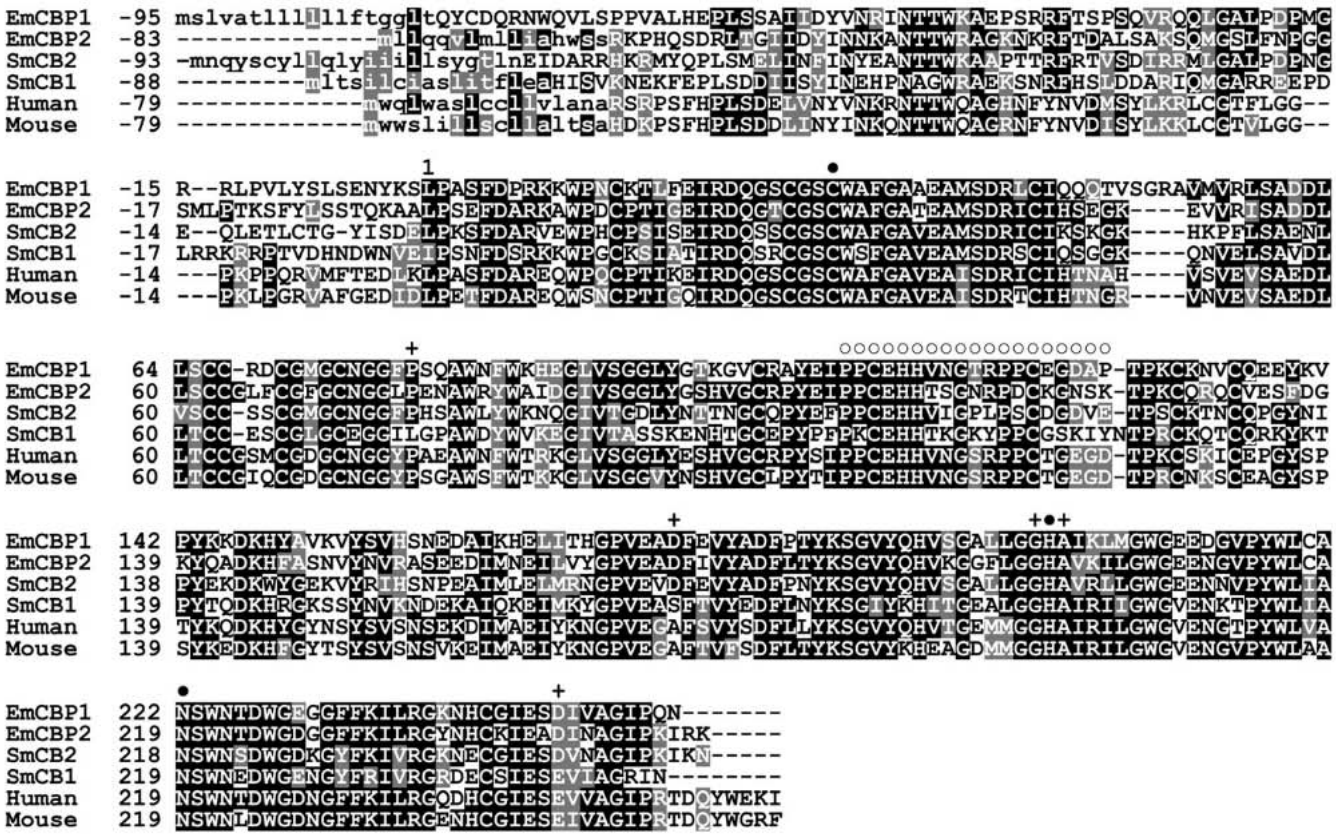

Fig. 1. Comparison of the deduced amino acid sequences of EmCBP1 and EmCBP2 with other cathepsin B enzymes. The alignment was generated using Clustal W server (http://www.ch.embnet.org/software/ClustalW.html) together with BOXSHADE server (http://www.ch.embnet.org/software/BOX_form.html). Gaps were introduced to maximize the alignment. Conserved residues are highlighted: identical, similar and unrelated residues with black, gray, and white backgrounds. Predicted signal sequence is written in lower case and closed circles (d) represent active site residues. Amino acid residues forming substrate binding pockets (McGrath, 1999) are indicated by plus signs (+), and the occluding loop unique to cathepsin B is indicated by open circles (s). Aligned amino acid sequences are *Schistosoma mansoni* cathepsin B (SmCB2, AJ312106), *Schistosoma mansoni* cathepsin B (SmCB1, AAA29865), human cathepsin B (Human, NP_001899), and mouse cathepsin B (Mouse, 1701299A).

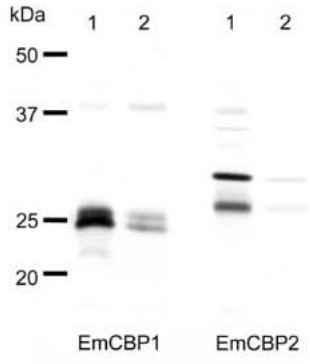

Fig. 2. Immunoblot analyses of *E. multilocularis* metacestodes extracts and ES products. Extracts of *E. multilocularis* metacestodes (lanes 1), and ES products (lanes 2) were probed with anti-EmCBP1 (left) and anti-EmCBP2 (right) monoclonal antibody. Molecular size markers are indicated on the left.

and 25.5 kDa in lysate and ES products, and anti-EmCBP2 monoclonal antibody recognized proteins of 27.0 and 29.9 kDa in lysate and ES products. Isotype-matched negative control monoclonal antibody did not bind to any of these bands (data not shown).

Furthermore, immunohistochemical studies were performed to investigate the localizations of EmCBP1 and EmCBP2 in metacestode. As shown in Fig. 3, the germinal layer, the brood capsule, and the protoscolex were stained. No signals were obtained for the acellular laminated layer of parasite.

3.3. Expression of EmCBP1 and EmCBP2 in yeast

To generate functional peptidases for *in vitro* studies, recombinant EmCBP1(yEmCBP1) and EmCBP2 (yEmCBP2) were expressed in yeast using the *P. pastoris* system and the α-pheromone signal sequence for extracellular secretion. The culture supernatant was collected after 3 days of cultivation and was 20-fold concentrated. The hydrolysis activity of the supernatant treated with and without pepsin in the presence of a reducing agent L-cysteine against Z-Phe-Arg-MCA was tested to determine optimal activation conditions of recombinant enzymes before purification (Fig. 4A). yEmCBP1 was activated at pH 4.5 after 1 h without the pepsin treatment. In contrast, removal of pro-region by pepsin was required for activation of yEmCBP2 (Fig. 4A). The activated recombinant enzyme purified by cation-exchange chromatography as a single peak was analyzed by SDS–PAGE followed by Coomassie Blue staining and immunoblotting (Fig. 4B and C). The purified yEmCBP1 migrated as a broad band between 25 and 50 kDa, and the purified yEmCBP2 migrated as a single band of approximately 27.0 kDa. Treating yEmCBP1 with PNGase F converted the broad band to two bands at 30 and 25.6 kDa (Fig. 4C, lanes 1 and 2), whereas no change in size of yEmCBP2 was observed (Fig. 4C, lanes 3 and 4), which indicated that yEmCBP1 was glycosylated. Analyses using the probe, biotin-Z-Phe-Ala-FMK, able to label specifically active cysteine peptidases revealed that in yEmCBP1 enzymes ranging from 30 to 50 kDa, detected as a 30 kDa band after treated with PNGase F, are active (Fig. 4D, lanes 1 and 2). No active enzyme bands except the 27-kDa enzyme were detected in yEmCBP2 (Fig. 4D, lanes 3 and 4). The labelling of active enzymes with biotin-Phe-Ala-FMK failed by pre-treatment with a cysteine inhibitor, E64 (data not shown).

3.4. Activity of yEmCBP1 and yEmCBP2 against peptidyl-MCA substrates

The substrate specificity of the yEmCBP1 and yEmCBP2 was characterized by using several peptide substrates varying at P2 position (Fig. 5). yEmCBP1 preferred substrates with Phe > Val > Leu at P2 position at an acidic pH optimum of 5.5. Substrate with Pro or Arg at P2 position was also hydrolyzed, but less efficiently. The pH optimum for hydrolyzing substrate with Arg at P2 was shifted to 7.5. yEmCBP2 showed similar features to those of yEmCBP1 except that a marked shifting of the pH optimum for hydrolyzing substrate with Arg at P2 was not observed. yEmCBP2 hydrolyzed peptidyl-MCA substrates more efficiently than yEmCBP1.

Kinetic parameters for hydrolysis of Z-Phe-Arg-MCA (suitable substrate for cathepsin L and B) and Z-Arg-Arg-MCA (cathepsin B-selective substrate) were summarized in Table 1. yEmCBP1 and yEmCBP2 had greater k_{cat}/K_m value for Z-Phe-Arg-MCA over Z-Arg-Arg-MCA. Difference in k_{cat}/K_m values between two substrates for yEmCBP1 and yEmCBP2 was 93- and 137-fold, respectively.

3.5. Inactivation kinetics of yEmCBP1 and yEmCBP2

The kinetics of the pH-induced inactivation of yEmCBP1 and yEmCBP2 were studies at pH 6.5, 7.0, and 7.5, and the reaction between enzymes and substrates (Z-Phe-Arg-MCA) was monitored continuously (Fig. 6 and Table 2). The inactivation of yEmCBP1 at pH 6.5 or 7.0 was not observed during monitoring period and its half-time at pH 7.5 was approximately 318 s. The half-time of yEmCBP2 shortened, from approximately 1689 to 10 s, with increasing pH.

3.6. Degradation of macromolecules by yEmCBP1 and yEmCBP2

To investigate the ability of yEmCBP1 and yEmCBP2 to degrade macromolecules, protein digestion analyses were performed (Fig. 7). In these studies, human IgG and human serum albumin

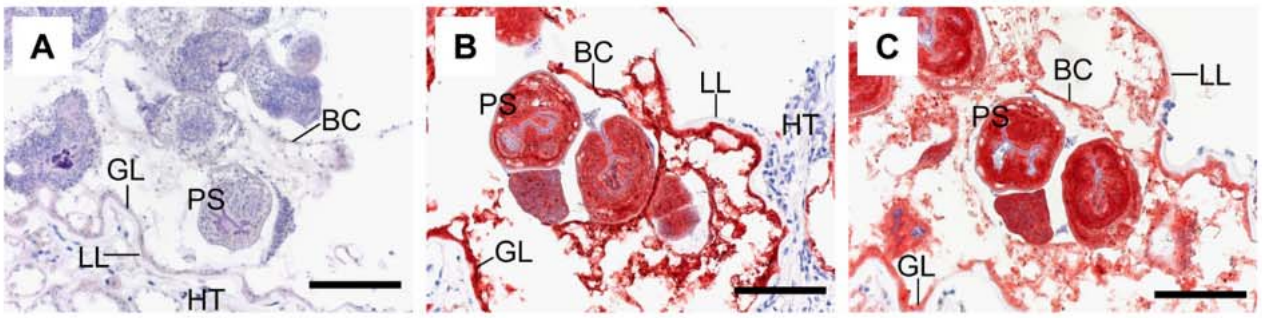

Fig. 3. Immunohistochemical detection of EmCBP1 and EmCBP2 in *E. multilocularis* metacestodes. Parasite tissues (A, B, and C) were isolated and paraffin-sections were produced. The sections were probed with anti-EmCBP1 (B), anti-EmCBP2 (C) and isotype-matched negative control (A) monoclonal antibody. The following structures are indicated: PS, protoscolex; GL, germinal layer; BC, brood capsule; LL, laminated layer; HT, host tissue. Scale bar = 100 μm.

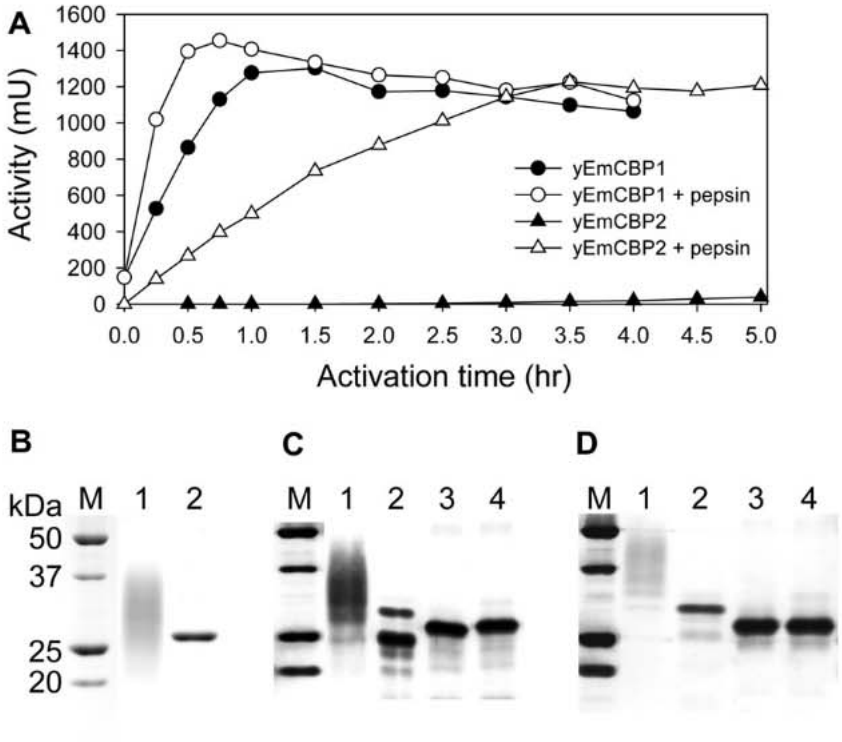

Fig. 4. Expression, purification and active-site labelling of yEmCBP1 and yEmCBP2. (A) Time-dependent activations with and without the pepsin treatment. Aliquots were withdrawn from the incubation mixture at the indicated time points, and the activities were monitored with 50 μM Z-Phe-Arg-MCA. (B) Purified recombinant enzymes were subjected to SDS–PAGE and stained with Coomassie blue. Lane 1, yEmCBP1; lane 2, yEmCBP2. (C) Immunoblot analyses of purified recombinant enzymes before (lanes 1 and 3) and after (lanes 2 and 4) deglycosylation by the treatment with PNGase F. yEmCBP1 (lanes 1 and 2) and yEmCBP2 (lanes 3 and 4) were detected by using monoclonal antibody specific for each protein. (D) Detection of active recombinant enzymes by labelling of purified recombinant proteins with the cysteine peptidase-specific probe, biotin-Phe-Ala-FMK. After labelling, aliquots of recombinant enzymes were treated with (lanes 2 and 4) and without (lanes 1 and 3) PNGase F. yEmCBP1 (lanes 1 and 2) and yEmCBP2 (lanes 3 and 4) were detected with alkaline phosphate-conjugated streptavidin. Molecular size markers are indicated on the left.

as humoral molecules and type I and type IV collagens and fibronectin as extracellular matrix molecules were chosen. All protein substrates used in this study were readily hydrolyzed by yEmCBP1 and yEmCBP2. All degradations of protein substrates were completely inhibited by adding a cysteine peptidase inhibitor, E-64 (data not shown).

4. Discussion

Numerous studies have demonstrated that cysteine peptidases from protozoa, trematode and nematode parasites are involved in various functions including nutrient uptake, disruption of the immune system, invasion and penetration into host tissues, which leads us strongly to consider them as a likely target for the chemotherapy (reviewed by Sajid and McKerrow, 2002; Dalton et al., 2003; Caffrey et al., 2004; Rosenthal, 2004; McKerrow et al., 2006; Robinson et al., 2008; Smooker et al., 2010). By contrast, few characterizations of peptidases including cysteine peptidases of cestodes *E. multilocularis* and *Echinococcus granulosus* have been described (McManus and Barrett, 1985; Marco and Nieto, 1991; Sako et al., 2007). In this study, two cathepsin B-like cysteine peptidases, EmCBP1 and EmCBP2, from *E. multilocularis* metacestode were identified, functionally expressed and characterized.

Sequencing analyses revealed that EmCBP1 and EmCBP2 have a catalytic triad (Cys, His, and Asn) and an oxyanion hole (Menard et al., 1991) those are characteristic features of clan CA family C1 cysteine peptidase. The occluding loop that is responsible for peptidyldipeptidase activity (Musil et al., 1991) and is a feature distinguishing cathepsin B from other cysteine peptidases is also conserved. RT-PCR analyses using EmCBP1 and EmCBP2-specific primer, in addition to the fact that EmCBP1 and EmCBP2 were found in the database of *E. multilocularis* whole genome project (http://www.sanger.ac.uk/resources/downloads/helminths/echinococcus-multilocularis.html), demonstrated that the genes obtained were originated from *E. multilocularis*, not from mouse used for preparation of parasite materials (data not shown).

Immunoblot and immunohistochemical experiments demonstrated that EmCBP1 and EmCBP2 were expressed in the germinal layer, the brood capsule and the protoscolex of larva and that some portions of both enzymes were secreted. The sizes, 24.5 and 25.5 kDa, of proteins detected by anti-EmCBP1 monoclonal antibody were smaller than the predicted size, 28.4 kDa. Cathepsin B is synthesized as an inactive 43 kDa pro-form and is processed to a single-chain form (31 kDa) or a two-chain form (heavy chain of 25 kDa and light chain of 5 kDa) to be activated (Towatari et al., 1979). It is possible that EmCBP1 consists of a heavy chain and light chain linking by a disulphide bond, and the protein bands detected by monoclonal antibody might be derived from the heavy chains. Anti-EmCBP2 monoclonal antibody recognized protein of 27.0 and 29.9 kDa in lysate and ES products. The latter protein might be intermediate forms of proenzyme. Determinations of N-terminal amino acid sequences of purified native EmCBP1 and EmCBP2 must be carried out.

The expressions of the active recombinant EmCBP1 and EmCBP2 were conducted by the use of the *P. pastoris* expression system. yEmCBP1 was successfully activated at pH 4.5 and purified. Because of the existence of one N-glycosylation site in mature enzyme, yEmCBP1 was glycosylated and detected as a broad band with 25–50 kDa range in size after purification. Some portion of yEmCBP1, detected as a 25.6 kDa-protein by immunoblot analysis after treated with PNGase F, could not be labelled efficiently with active enzyme-specific probe, biotin-Phe-Ala-FMK, which indi-

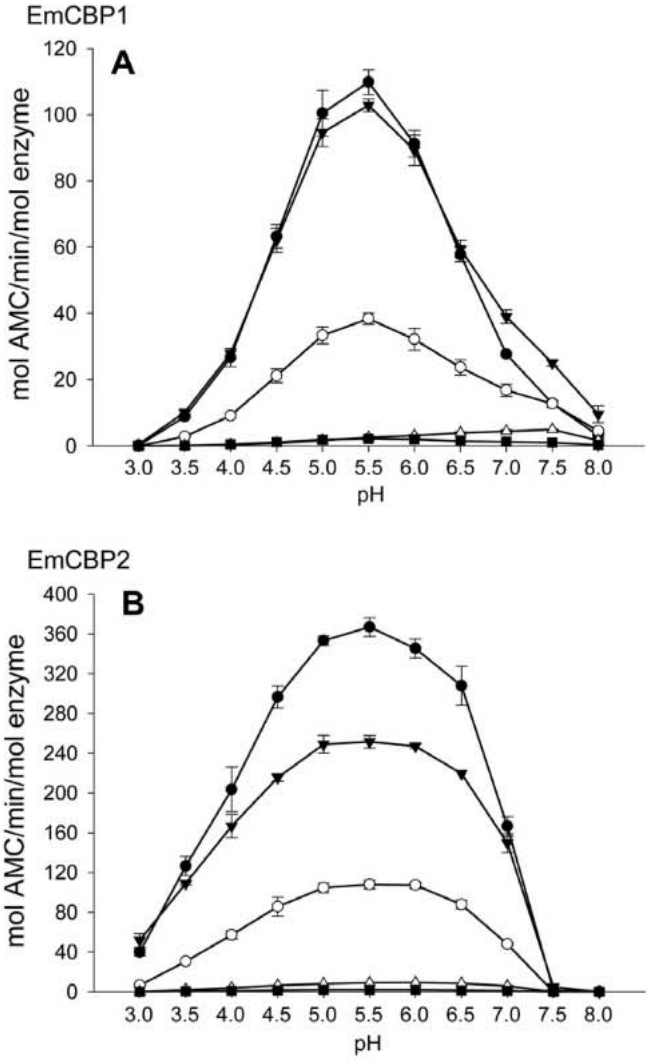

Fig. 5. pH optima and S2 subsite specificity of yEmCBP1 and yEmCBP2. Five substrates, Z-Phe-Arg-MCA (closed circles), Z-Val-Val-Arg-MCA (closed triangles), Z-Leu-Arg-MCA (open circles), Z-Gly-Pro-Arg-MCA (closed squares), and Z-Arg-Arg-MCA (open triangles) were tested at a final concentration of 2 μM. The standard deviation of three experiments is indicated.

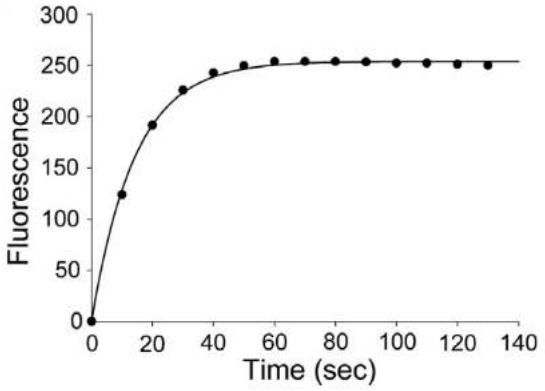

Fig. 6. Progress curve for the inactivation of yEmCBP2 at pH 7.5. Substrate, Z-Phe-Arg-MCA, was tested at a final concentration of 50 μM, and progress of the reaction was monitored continuously by the fluorescence of the released products. The solid line is the theoretical first-order curve calculated using Eq. 1.

Table 2
Effect of pH on the rate of inactivation of yEmCBP1 and yEmCBP2.[a]

	pH	$10^3 \times k_{obs}$ (s^{-1})	$t_{1/2}$ (s)
yEmCBP1	6.5	Nob[b]	Nob
	7.0	Nob	Nob
	7.5	2.18 ± 0.02	317.69 ± 3.40
yEmCBP2	6.5	0.41 ± 0.01	1689.29 ± 61.11
	7.0	6.79 ± 0.48	102.32 ± 7.20
	7.5	70.52 ± 2.23	9.83 ± 0.31

[a] The best estimates for the observed inactivation rate constant, k_{obs}, are given by nonlinear regression analysis. Corresponding half-lives were calculated using following relation ship: $t_{1/2} = \ln 2/k_{obs}$. Inactivation was investigated in the presence of 50 μM Z-Phe-Arg-MCA.
[b] Nob = not observed during incubation.

cated that they were inactive enzyme. This may be due to miss-folding, the oxidation, or the autodegradation of the mature enzyme during expression and purification. Activation of yEmBP2 was unsuccessful under the same condition of yEmCBP1 activation. Alternative activation condition in the presence of negatively charged glycosaminoglycan with dextran sulfate which facilitates autocatalytic activation (Barlic-Maganja et al., 1998) was tested but resulted in unsuccessful (data not shown). However, active yEmCBP2 could be obtained by *trans*-processing with pepsin, resulting in a single 27 kDa-protein.

In cysteine peptidases belonging to clan CA, the S2 subsite is substantial substrate-binding pocket for determination of substrate specificity (McGrath, 1999). The substrate specificity of the yEmCBP1 and yEmCBP2 was characterized by the use of several peptide substrates varying at P2 position. Both enzymes preferred substrates with Phe > Val > Leu at P2 position at an acidic pH optimum of 5.5, and cathepsin B-selective substrate Z-Arg-Arg-MCA was less hydrolyzed. Kinetic parameters for hydrolysis of Z-Phe-Arg-MCA and Z-Arg-Arg-MCA revealed that there were 93- and 137-fold-differences in k_{cat}/K_m values for yEmCBP1 and yEmCBP2, respectively. Similar preference has been observed in a cathepsin B isoform (SmCB2) of *Schistosoma mansoni*, not other isoform (SmCB1) (Caffrey et al., 2002). By contrast, the difference reported for mammalian cathepsin B is smaller than 10-fold (Hasnain et al., 1992; Wang et al., 1998). EmCBP1, EmCBP2 and SmCB2 have a negatively charged residue Asp at position 173 (mouse cathepsin B

Table 1
Kinetic parameters for hydrolysis of peptidyl-MCA substrates by yEmCBP1 and yEmCBP2.[a]

	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)
yEmCBP1	Z-Phe-Arg-MCA	20.45 ± 1.23	33.23 ± 1.01	1626.71 ± 48.67
	Z-Arg-Arg-MCA	747.67 ± 41.15	13.05 ± 0.63	17.46 ± 0.11
yEmCBP2	Z-Phe-Arg-MCA	27.17 ± 0.82	71.68 ± 1.52	2638.40 ± 23.29
	Z-Arg-Arg-MCA	1124.47 ± 76.15	21.57 ± 1.05	19.19 ± 0.36

[a] The K_m and V_{max} values were calculated by a nonlinear regression analysis of substrate concentration versus peptidase velocity. The k_{cat} values were calculated from V_{max} and the molar concentration of active enzyme titrated with Z-Phe-Arg-MCA and cysteine peptidase inhibitor E-64.

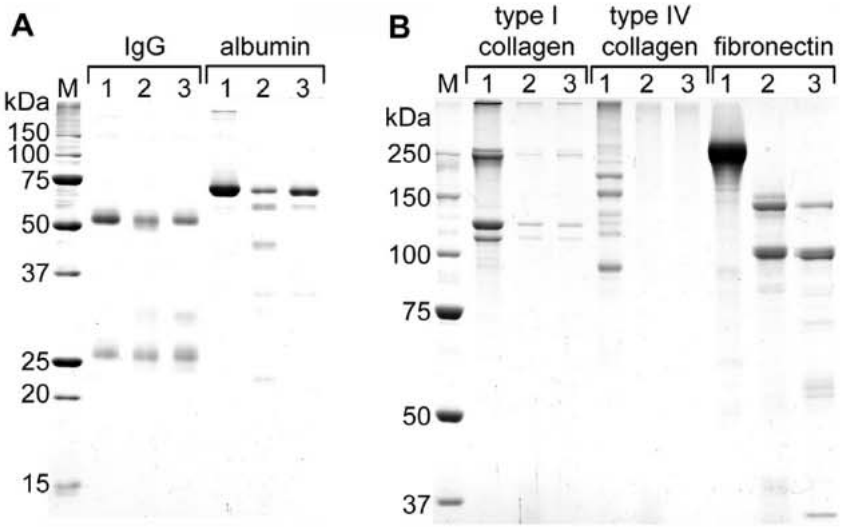

Fig. 7. Degradation of macromolecules by yEmCBP1 and yEmCBP2. Humoral molecules (A) and extracellular matrix molecules (B) were incubated with yEmCBP1 or yEmCBP2 at 37 °C for 4 h at pH 5.5. Protein substrate digests were subjected to SDS–PAGE and degradation products were visualized by Coomassie Blue staining. Each substrate was incubated with none of enzyme (lanes 1), yEmCBP1 (lanes 2), and yEmCBP2 (lanes 3). Molecular size markers are indicated on the left.

numbering) involving in S2 subsite formation, whereas mammalian cathepsin B from human, mouse, rat, and bovine and SmCB1 have an uncharged residue, Ala and Ser, respectively, without other substantial differences. Therefore, the possibility that the negatively charged residue Asp participates in the substrate preference is raised. Further analyses by using recombinant enzymes in which Asp is replaced with Ala are necessary to confirm this possibility.

The facts that at acidic condition yEmCBP1 and yEmCBP2 were stable, active and had broad specificity against host proteins including immunoglobulin, albumin, collagens, and fibronectin, suggested that these enzymes are lysosomal enzymes and are involved in protein digestion for parasites' nutrition. Additionally, the possibility that yEmCBP1 and yEmCBP2 act as extracellular enzymes was raised since both enzymes were detected in ES products. However, yEmCBP1 and yEmCBP2, especially latter, were unstable at neutral or slightly alkaline pH close to physiological pH similarly to mammal papain-like cysteine peptidases except for cathepsin S (Turk et al., 2000). Since the echinococcal cyst fluid has a neutral pH, EmCBP1 and EmCBP2 secreted into cyst fluid might lose their enzymatic activity. In contrast, EmCBP1 and EmCBP2 secreted outside of parasite cysts might not lose their enzymatic activity, because it is known that the host inflammatory responses can lead to tissue acidification (Kellum et al., 2004) and cathepsin B secreted from some kind of tumor cells becomes stable at alkaline pH by interacting with heparin and heparan sulfate (Almeida et al., 2001; Roshy et al., 2003). The activities of cysteine peptidases secreted into host tissues to degrade extracellular matrix molecules, e.g., collagen and fibronectin, have been described in several nematode and trematode parasites (Berasain et al., 1997; Rhoads and Fetterer, 1997; Smooker et al., 2010), and these characters seem to be implicated in migration of parasite through host tissues. Because the larva of *E. multilocularis* infiltrates and proliferates indefinitely by exogenous budding of the cellular germinal layer (Thompson, 1995), the ability of EmCBP1 and EmCBP2 to degrade extracellular matrix molecules might be involved in pathogenesis of *E. multilocularis*. Inhibition analyses of EmCBP1 and EmCBP2 by specific inhibitors or genetic knock out of enzymes would be needed to confirm such a role of peptidases *in vivo*.

In conclusion, we identified and characterized two novel cathepsin B-like peptidases from *E. multilocularis* metacestodes. The enzymes may play a primary role in protein digestion for parasites' nutrition. Thus, the inactivation of these enzymes may impair the survival of the parasite in the host. Further studies are needed to provide a greater understanding of the biological significance of EmCBP1 and EmCBP2 in parasite–host interactions.

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