

Localization of Eimeripain, an *Eimeria tenella* Cathepsin B-Like Cysteine Protease, during Asexual and Sexual Intracellular Development in Chicken Ceca

Makoto MATSUBAYASHI¹⁾, Takeshi HATTA¹⁾, Takeharu MIYOSHI¹⁾, ANISUZZAMAN¹⁾, Kazumi SASAI²⁾, Kayoko YAMAJI¹⁾, Kameo SHIMURA¹⁾, Takashi ISOBE¹⁾, Kiyoshi KITA³⁾ and Naotoshi TSUJI^{1)*}

¹⁾National Institute of Animal Health, National Agriculture and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

²⁾Department of Veterinary Internal Medicine, Division of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58 Rinku Orai-kita, Izumisano, Osaka 598-8531, Japan

³⁾Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

(Received 14 October 2013/Accepted 6 December 2013/Published online in J-STAGE 20 December 2013)

ABSTRACT Hemorrhagic diarrhea in poultry is caused by *Eimeria tenella*, the most pathogenic avian coccidian parasite, and new approaches to treat the disease are continually being sought. Although eimeripain, a cathepsin B-like cysteine protease from *E. tenella*, has recently been identified as a novel anticoccidial drug target, its localization during the intracellular development of parasites remains unclear. Here, we demonstrate the expression of eimeripain during asexual and sexual development of *E. tenella* *in vivo*. Promature eimeripain was detected only in the early immature second generation of schizonts. In contrast, the mature eimeripain was most strongly detected in the middle-sized immature second generation of schizonts. Both promature and mature eimeripain disappeared depending on the maturation level of second generation of schizonts, but were strongly expressed again in the third generation of schizonts. In the sexual stage, both promature and mature eimeripain were detected in the cytoplasm of micro- and macro-gametocytes and zygotes, but expression became weak in zoites forming oocysts. Collectively, our findings suggest that eimeripain might play a key role in the differentiation of intracellular zoites in the ceca and could be an interesting candidate to develop a novel, effective anti-coccidian drug.

KEY WORDS: cysteine protease, *Eimeria tenella*, eimeripain, *in vivo* development.

doi: 10.1292/jvms.13-0509; *J. Vet. Med. Sci.* 76(4): 531–537, 2014

Eimeria tenella is widely considered to be the most pathogenic of chicken *Eimeria* spp. due to its motility. The complex and monoxenous life cycle of *E. tenella* is divided into intestinal and environmental stages. Oocysts excreted with host feces undergo sporulation in the external environment and form four sporocysts containing two sporozoites. Following ingestion of the sporulated oocysts by chicken, sporozoites released in the intestines invade host cells, resulting in three asexual (development of schizonts for formation of merozoites) and one sexual (development of micro- and macro-gametocytes and formation of zygotes) development phases [4]. Consequently, the host presents bloody diarrhea, a decrease in weight gain and eventual death. Although prophylactic chemotherapy and vaccination using live vaccines are viable options, the increase in resistant parasites and the difficulty involved in production of live attenuated parasites underline the necessity to find alternative target molecules and drugs.

Recently, eimeripain, a cathepsin B-like cysteine protease

of *E. tenella*, has been enzymatically characterized, and some lead compounds against eimeripain have been identified as novel anticoccidial drugs [8, 10]. However, these analyses were focused only on the extracellular stage of *E. tenella* life cycle, including sporozoites. No evidence exists that this enzyme could be expressed in asexual or sexual developmental stages, even though effective antiparasite drugs preferentially target molecules expressed at the intracellular stages in hosts. These enzymes have a prodomain and a mature domain, and the prodomain needs to be cleaved for full activity of the enzyme. In the present study, we analyzed native eimeripain as a promature and mature enzyme during the intracellular stages in the chicken ceca at the mRNA and protein levels to resolve this important question.

MATERIALS AND METHODS

Parasites and chicks: The NIAH strain of *E. tenella*, which is virulent and maintained at the Laboratory of Parasitic Diseases, National Institute of Animal Health (Tsukuba, Japan), was used throughout this study. The parasites were maintained by passage in 2- to 3-week-old chicks (Nisseiken, Tokyo, Japan). The chicks were housed in wire-floored cages in coccidian-free rooms and with free access to feed and water that contained no anticoccidial drugs or antibiotics. The animals were treated in accordance with protocols approved by the Animal Care and Use Committee, NIAH (Approval nos. 11-026 and 12-029). They were orally inocu-

*CORRESPONDENCE TO: TSUJI, N., Laboratory of Parasitic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan. e-mail: tsujin@affrc.go.jp

©2014 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/3.0/>>.

lated with 2×10^4 oocysts, and feces were collected after 6–8 days. Oocysts were purified from feces using the sugar flotation method.

Preparation of developmental zoites: The purified *E. tenella* oocysts were incubated for various time periods (0 hr, 48 hr and 144 hr) at 28°C in 2.5% potassium dichromate (Wako, Osaka, Japan), treated with sodium hypochlorite (Nacalai Tesque, Kyoto, Japan) for 20 min at 4°C and then washed with phosphate-buffered saline (PBS). This chemical treatment was repeated twice, followed by washing with PBS 5 times, and processed for further analysis as described below.

For the purification of sporozoites, the sporulated oocysts were broken by vortexing with glass beads for 1–2 min to release sporocysts. The sporocysts were treated in excystation medium, 0.25% (w/v) trypsin (Merck, Darmstadt, Germany) and 1% (w/v) taurodeoxycholic acid (Sigma, St. Louis, MO, U.S.A.) in Hanks' balanced salt solution (Sigma), pH 7.4, at 41°C in a 5% CO₂ incubator for 90–100 min. The sporozoites were obtained by purification involving two steps, namely centrifugation at 450 g for 2 min according to a modification of a previously described method [12] and a filtration protocol using 595 filter paper circles [5].

Merozoites and schizonts of the second generation were purified by centrifugation using Percoll (GE Healthcare Life Sciences, Piscataway, NJ, U.S.A.) as reported previously [2]. Briefly, the chicks were orally inoculated with 2×10^4 oocysts, and the infected ceca were removed at 96 to 108 hr post-inoculation after euthanasia of animals according to protocols approved by the Animal Care and Use Committee, NIAH (Approval nos. 11-026 and 12-029). After removal of the contents and washing with PBS, the ceca were cut into less than 5 mm pieces and filtered by a wire mesh. These steps were performed on ice. The filtered homogenate was mixed with 100% Percoll to make a 30% Percoll-PBS homogenate and layered over 50% Percoll-PBS, which were finally layered over PBS. A total of three layers was prepared and centrifuged at 1,300 g for 15 min. After centrifugation, a high population of schizonts was seen floating on the 30% Percoll-PBS; merozoites were concentrated on the 50% Percoll-PBS, while erythrocytes were collected as a red pellet at the bottom. These zoites were aspirated and washed with PBS three times. The merozoites were filtered using 595 filter paper circles as described above.

Sections of the infected ceca were prepared as described previously [4]. Briefly, 2-week-old chicks in groups of three were inoculated with 2×10^4 oocysts, and the infected ceca were removed at 72 hr, 96 hr, 120 hr, 144 hr and 168 hr post-inoculation and then fixed. The ceca embedded in paraffin were cut at a thickness of 4 μ m.

Cloning and sequencing of eimeripain: The gene coding for eimeripain was partially identified from the *E. tenella* Gene Index Database (Sequence No. CD658426). For cloning of the full-length eimeripain, 5' and 3' rapid amplification of cDNA ends (RACE) was performed using the SMARTer RACE cDNA Amplification Kit (Takara, Otsu, Japan) according to the manufacturer's protocol using these primers: EtCathep-F1; CAA CTT CGA CCA CGT GCC CAT TTC

TCT T for the 5' end and EtCathep-F14; TAC TGG CTA GCT GTG AAC AGC TGG for the 3' end. The putative signal sequence of eimeripain was analyzed using the prediction server SignalP V4.1 [6]. By sequence analysis of RACE products, 1,536 bp of eimeripain was cloned as the complete coding region. All sequences were completely identical with those previously reported [8, 10]. This molecule also contained approximately 410 bp and 1,180 bp non-coding regions at the 5' and 3' ends, respectively, consisting of a 512 amino acid protein, with a signal peptide of 21 hydrophobic amino acids, a predicted molecular weight of 54.68 kDa and a pI of 5.53 (excluding signal peptide) using PeptideMass [14].

Expression of eimeripain: The open reading frame (ORF) of eimeripain was amplified by PCR from a cDNA of sporulated oocysts using a set of primers [forward: ReEtCathep-F1 (CGG GGT ACC CCG ATG CCC TCC GAT GAT TTG GGC), reverse: ReEtCathep-F2 (GGG GTA CCC CTC ATA GGT CCT GCG CTG ACG G)] containing Kpn I restriction sites. PCR was performed for 5 min at 94°C followed by 30 cycles of 30 sec at 94°C, 30 sec at 63.4°C and 2 min at 72°C and a final elongation at 72°C for 10 min. The PCR product and the vector pTrcHisB (Invitrogen, Carlsbad, CA, U.S.A.) were digested by Kpn I restriction enzymes. The purified PCR product was inserted into the Kpn I sites of the vector pTrcHisB using DNA ligation kit (Takara). The inserted eimeripain sequence was confirmed using internal primers. The resultant plasmid was transformed into competent cells of *Escherichia coli* Top10F' strain (Invitrogen) following the conventional method. The expression of eimeripain in *E. coli* with a Polyhistidine-tag was performed according to the procedure described by Tsuji *et al.* [13]. Briefly, the transfected cells were allowed to grow in SOB medium (BD, Franklin Lakes, NJ, U.S.A.) containing 50 mg ampicillin/ml at 37°C. To induce recombinant protein expression, isopropyl-b-D-thiogalactopyranoside (IPTG) was added at 1 mM concentration, and the culture was grown for an additional 4 hr at 37°C. The culture was then centrifuged at 10,000 g for 20 min and 4°C, and then, the pellet was resuspended in lysis buffer (20 mM sodium phosphate and 500 mM sodium chloride, pH 7.8) with egg white lysozyme (100 μ g/ml). The suspension was sonicated on ice with an ultrasonic processor (Taitec, Saitama, Japan) followed by freezing and thawing. After three cycles of this treatment, the *E. coli* lysate was centrifuged at 23,900 g for 30 min at 4°C, and the supernatant was collected. As a result, eimeripain was successfully expressed in *E. coli*, harvested as a soluble protein from bacterial cultures, separated on 12.5% SDS-PAGE gel and detected as an approximately 60 kDa fusion protein by Western blotting using an anti-His monoclonal antibody (Nacalai Tesque) as described below. The recombinant protein was purified using ProBond™ resin (Invitrogen) as described by the manufacturer. Purified recombinant eimeripain was separated on 12.5% SDS-PAGE gel and detected as a single band by staining with 0.2% Coomassie Brilliant Blue R-250 (Wako) (Fig. 1). Protein concentration was calculated using the BCA protein assay kit (Pierce, Rockford, IL, U.S.A.), and protein was stored at –20°C until further use.

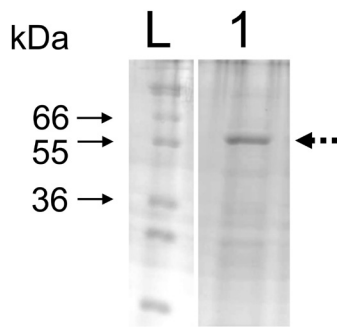


Fig. 1. SDS-PAGE of fusion-protein recombinant eimeripain after purification of ProBond™ resin. L: molecular weight marker, 1; recombinant eimeripain.

Reverse transcription polymerase chain reaction (RT-PCR): For RT-PCR, six samples of unsporulated oocysts, sporulated oocysts incubated for 48 hr and 144 hr, and purified sporozoites, merozoites and schizonts were used. They were washed in PBS and submerged in RLT lysis buffer (Qiagen, Hilden, Germany). Total RNA from each sample was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Single stranded cDNA for mRNA was prepared using the Takara RNA PCR Kit (AMV) Ver.3.0 (Takara) following the manufacturer's instructions. Four hundred nanograms of total RNA was used for RT before PCR. As a control, RNA from chicken cells purified by the protocol for schizonts and merozoites using non-infected ceca were used, because chicken cells might contain purified schizonts or merozoites. The synthesized cDNAs were used for PCR using eimeripain [forward: EtCathep-F5 (ATT CTG CGA GAT GTA GAG AAG GAT ATT), reverse: EtCathep-R5 (CTG GAT CTG AAT AGA AAG AAA GGT AAG T)] (499bp) and *E. tenella* actin (350bp) [9] specific oligonucleotides. As an additional control, RNA samples treated with the same protocol for synthesizing cDNA without transcriptase were used. PCR was performed for 5 min at 94°C followed by 40 cycles of 30 sec at 94°C, 60 sec at 53°C and 60 sec at 72°C and finally elongation at 72°C for 10 min. The PCR product was subjected to electrophoresis on a 1.5% agarose gel.

Polyclonal antisera against eimeripain: An antiserum against the eimeripain was raised by immunizing BALB/c mice subcutaneously with 100 µg of purified recombinant protein emulsified with complete Freund's adjuvant (Difco Laboratories, Detroit, MI, U.S.A.), followed by booster immunizations of 50 µg at 2 weeks apart using the same route five times. The immunized mice were sacrificed 1 week after the last booster, and serum was collected and stored at -20°C for further use.

The peptide CGGEPKVPNDKNAS, amino acids 257 to 270 of the mature domain of eimeripain as previously reported [10], were synthesized and coupled to keyhole limpet hemocyanin (KLH) (Pierce). This synthetic peptide was used to raise polyclonal antibodies against the mature domain of

eimeripain in a Japanese white rabbit by subcutaneous injection of 400 µg of the antigen emulsified with complete Freund's adjuvant (Difco Laboratories). The protocol was the same as that described above, except for the booster immunizations using 200 µg of antigen.

Western blotting: The sporulated oocysts (48 hr incubation) and purified sporozoites, merozoites and schizonts were resuspended in PBS. They were freeze-thawed five times, sonicated in an ice bath with the ultrasonic processor (Taitec) and centrifuged at 600 g for 5 min. The supernatant was aliquoted and cryopreserved at -80°C until use. The concentration of crude antigens was determined by BCA protein assay kit (Pierce). These parasite lysates (10–20 µg) and the purified recombinant protein of eimeripain (2 µg) were separated through 12.5% SDS-PAGE gels under reducing conditions, and the proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences). The membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) for 30 min and then incubated with polyclonal antibodies against the recombinant eimeripain or the peptide corresponding to the eimeripain mature domain (1:1,000) for 1 hr at room temperature. They were washed with TBS containing 0.05% Tween 20 (TBS-T) and then incubated with alkaline phosphate-conjugated goat anti-mouse or rabbit IgG (H + L) (Zymed, South San Francisco, CA, U.S.A.) as a secondary antibody for 1 hr. The membrane was washed again with TBS-T, and the bound antibody was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT, Promega, Madison, WI, U.S.A.).

Indirect immunofluorescence assay: The purified sporozoites and merozoites were applied to slide glass, air-dried and fixed by methanol. These prepared slides and sections of infected ceca were blocked with 10% goat serum (MP Biomedicals, Santa Ana, CA, U.S.A.) for 30 min, washed three times with 0.05% tween 20 in PBS (PBST) and then incubated with polyclonal antibodies (diluted 1:1,000) against recombinant eimeripain and eimeripain mature domain peptide for 1 hr. The slides were washed three times with PBST and then incubated with green fluorescence-labeled mouse and rabbit IgG secondary antibodies [Alexa Fluor®488 goat anti-(mouse IgG) (H + L) or anti-(rabbit IgG) (H + L); Invitrogen] for 1 hr. They were observed under a fluorescence microscope (Leica, Wetzlar, Germany). All steps were performed at room temperature.

RESULTS

Transcription of eimeripain: In the present study, the total RNA that was first extracted from several stages of *E. tenella* was subjected to RT-PCR analysis for transcription profiling of eimeripain. By successful amplification of an approximately 500 bp fragment, we confirmed that eimeripain was expressed in all examined stages, namely oocyst (0 hr, 48 hr and 144 hr after sporulation), sporozoite, schizont and merozoite stages, but not in samples of chicken cells or in any of the controls (Fig. 2A), although the expression between mRNA and protein did not always correlate. *E. tenella* actin

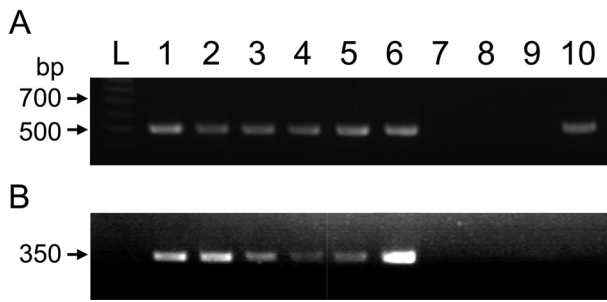


Fig. 2. Expression of eimeripain genes during the developmental stages of *E. tenella*. Detection of eimeripain (499 bp) (A) and *E. tenella* actin (350 bp) (B) specific RNA. L; DNA marker, 1; RNA from unsporulated oocysts, 2; from oocysts sporulated at 48 hr, 3; from oocysts sporulated at 144 hr, 4; from purified sporozoites, 5; from purified schizonts, 6; from purified merozoites, 7 and 8; from chicken cells separated on a 30%, and 50% Percoll layer using uninfected chicken ceca, respectively, 9; only PCR buffer, 10; genomic DNA from sporulated oocysts.

gene showed the same results with those of eimeripain (Fig. 2B), except for *E. tenella* genomic DNA.

Endogenous form of eimeripain: In order to identify the endogenous form of eimeripain, anti-eimeripain antibodies were prepared using recombinant eimeripain and a peptide corresponding to the mature domain of eimeripain. By Western blotting analysis, the two polyclonal antibodies generated against eimeripain recognized the recombinant eimeripain fusion protein as a 60 kDa band (Fig. 3A and 3B, lane 5), and thus, these epitopes were confirmed to be present within the amino acid sequence of eimeripain. Consequently, a 54 kDa band of promature eimeripain, previously reported [8], was detected in all stages of sporulated oocysts, sporozoites, merozoites and schizonts examined with the antiserum against recombinant eimeripain (Fig. 3A, lanes 1–4). A 33 kDa band, which has been reported as the active form of eimeripain [8], was recognized only in sporulated oocysts and schizonts (Fig. 3B, lanes 1 and 3). Unexpectedly, the 54 kDa band of promature eimeripain was also detected in schizonts (Fig. 3B, lane 3). These results show that antisera against recombinant eimeripain and eimeripain mature domain recognize promature and mature eimeripain, respectively, and that mature eimeripain is undetectable or does not exist in invasive zoites like sporozoites and merozoites, but only in sporulated oocysts and developmental schizonts.

Endogenous localization of eimeripain in invasive zoites: Promature eimeripain was detected in the cytoplasm of sporozoites and merozoites by incubating with the antiserum against recombinant protein (Fig. 4). They were observed as several dots, but not all over the entire region of sporozoite and merozoite bodies, although these reactivities were not strong. The antibody against the eimeripain mature domain did not show any reactivity with these zoites (data not shown).

Endogenous localization during asexual stages: During the second generation, in sections at 72 hr, 96 hr and 120 hr post-infection, promature eimeripain was detected only

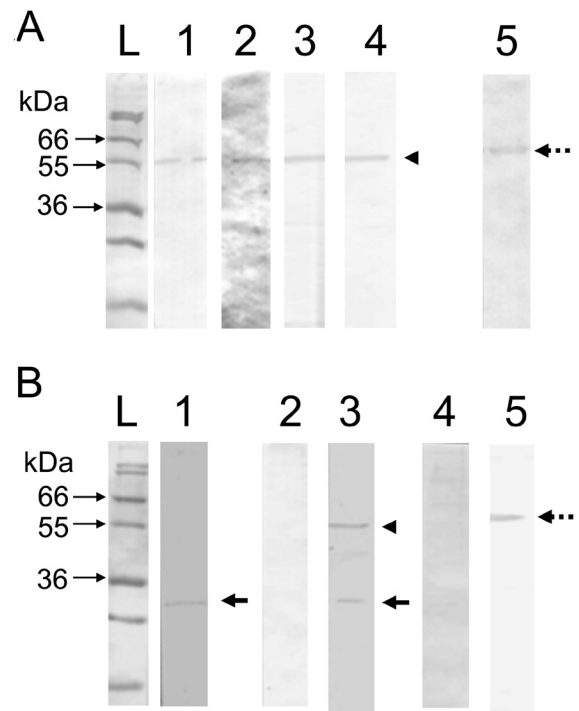


Fig. 3. Western blot analysis using antisera with anti-eimeripain (A) and anti-peptide against eimeripain mature domain (B). L: molecular weight marker, 1; sporulated oocysts, 2, purified sporozoites, 3 and 4; purified schizonts and merozoites of the second generation, 5; purified recombinant eimeripain. Arrows, arrowhead and arrows with broken line show 33 kDa mature eimeripain, 54 kDa promature eimeripain and 60 kDa recombinant eimeripain protein, respectively.

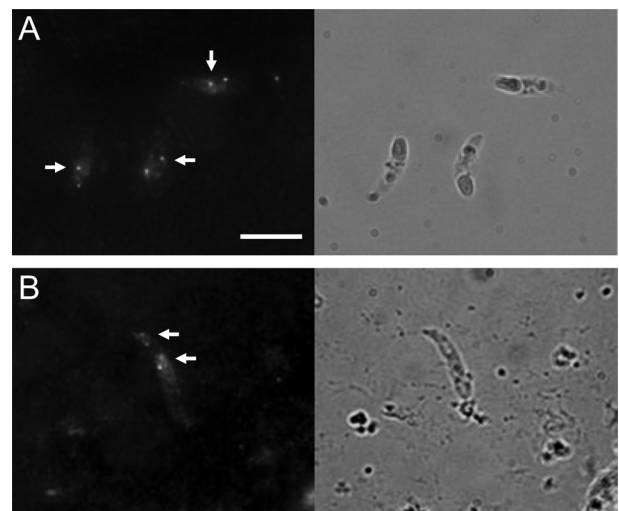


Fig. 4. Immunofluorescent staining of sporozoites (A) and merozoites (B) with antibody against promature eimeripain (arrows). Left and right figures show fluorescent and bright field photos. Scale bar is 10 μ m.

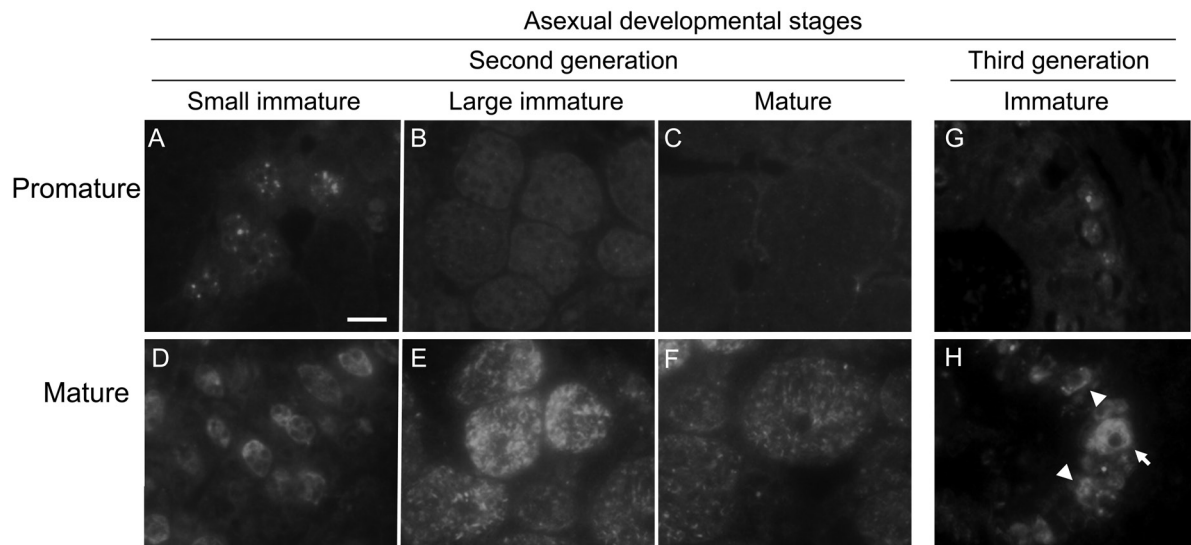


Fig. 5. Immunofluorescent staining of asexual stages, the second generation schizonts (A) and the third generation schizonts (B) with antisera against promature (upper photos) and mature (bottom photos) eimeripain. Figures A and D are small immature schizonts of the second generation, B and E are large immature schizonts, and C and F are mature schizonts. Figures G and H show the third generation schizonts. Arrowheads and arrows show smaller immature schizonts and larger immature ones of the third generation in Fig. H. Scale bar is 10 μ m.

in early schizonts at around 10 μ m in size, as constellations of dots in the cytoplasm (Fig. 5A); it was not detected in schizonts over 15 μ m (Fig. 5B and 5C). On the other hand, mature eimeripain was observed over the entire cytoplasm surrounding the nuclei in small immature schizonts and in developmental second-generation schizonts of middle size (around 20 μ m) with the strongest reactivity (Fig. 5D and 5E). In mature schizonts, this reactivity became weak and seemed to be present only between merozoites in the mature schizonts (Fig. 5F). In the third generation schizonts at 144 hr, reactivity of promature eimeripain was similar to that of the second generation, and this enzyme was observed as dots in the cytoplasm. Mature eimeripain was present as dots in the cytoplasm of small immature third schizonts and observed over the entire cytoplasm of large immature ones (Fig. 5G and 5H), but disappeared in mature third schizonts (data not shown).

Endogenous localization during the sexual stage: At the sexual stage, reactivities were found to be similar between promature and mature eimeripain (Fig. 6A and 6B) and different from those of the asexual stages described above. Both types of eimeripain were observed in the wall-forming bodies and cytoplasm of immature macro-gametocytes, only in the wall-forming bodies of mature macro-gametocytes, and across the cytoplasm of micro-gametocytes. In zygotes signals were present over the entire cytoplasm, except in the nuclei. At the oocyst formation stage, the final step of sexual development, which is seen as a distorted shape, eimeripain was weakly observed in the cytoplasm around the inner oocyst wall as spots (Fig. 6A and 6B, upper right figures).

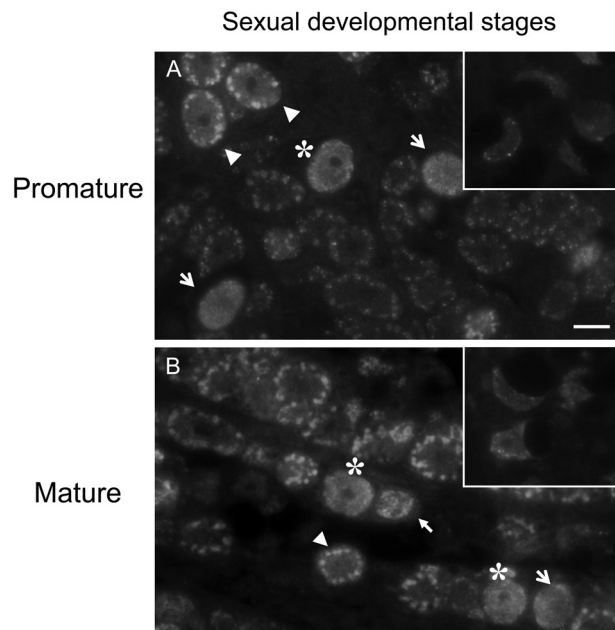


Fig. 6. Immunofluorescent staining of sexual stage with antibodies against promature (A) and mature (B) eimeripain. Arrowheads, arrows and asterisks show macro-gametocytes with a prominent wall forming body, micro-gametocytes and zygotes, respectively. Boxed figures are early zoites forming oocysts. Scale bar is 10 μ m.

DISCUSSION

Eimeripain has been proposed as a candidate drug target to be exploited against chicken coccidiosis in the future [8, 10]. In those studies, a panel of inhibitors was tested against the enzyme and three new inhibitors were identified [10]. Moreover, it was confirmed that eimeripain was expressed in the extracellular stage during sporulation [8]; however, no other life cycle stage was examined for its expression so far. In the present study, we generated two antisera against eimeripain; one recognized promature eimeripain, while the other recognized eimeripain by Western blotting. As a result, the eimeripain was detected as protein of promature at examined in all examined stages of sporulated oocysts, and purified sporozoites, merozoites and schizonts. While, mature protein was identified as 33 kDa molecule only at sporulated oocysts and schizonts, but not sporozoites and merozoites. Probably, the epitope recognized by anti-peptide sera against eimeripain mature domain might be masked by the modification of carbohydrate chain or protein folding in promature eimeripain before maturation. Interestingly, this polyclonal antibody also identified a 54 kDa band in schizonts, but did not react with sporozoites or merozoites. There is a possibility that purified schizonts represent many developmental stages from immature to mature second-generation schizonts, and thus, mature eimeripain of 54 kDa might be contained before complete maturation. These results suggest that mature eimeripain do not exist in invasive zoites, but only in sporulated oocysts and developmental schizonts.

By indirect immunofluorescence assay, the reactivities of promature eimeripain in sporozoites and merozoites were similar to that seen in transfected sporozoites, as previously reported [8], but mature eimeripain was not. In intracellular developmental stages, the promature enzyme was detected in the cytoplasm of early schizonts of the second and third generations, but disappeared during their respective developments. Meanwhile, mature protein was present in the entire cytoplasm, but later was not detected. Thus, the promature protein is present as dots in the early asexual schizonts and probably sporozoites and merozoites as well, and active eimeripain might spread throughout the cytoplasm during development or differentiation of schizonts. These findings tempt speculation that eimeripain might play a key role in the differentiation of intracellular zoites in the ceca in addition to the extracellular stage.

Cathepsins, which are cysteine proteases related to papain-like enzymes (clan CA, family C1), are major virulence factors expressed by apicomplexan parasites [1, 3, 11]. For instance, in *Toxoplasma gondii*, antisense or inhibitors of cathepsin B can block the invasion of tachyzoites into host cells and cause abnormal rhoptry morphology [7]. In higher eukaryotic cells, acidic cathepsin is important for protein processing and breakdown in lysosomes. However, the lysosomal system of *E. tenella*, like that of *Toxoplasma gondii*, has not been characterized. In our study, eimeripain was expressed in all examined intracellular stages at both mRNA and protein levels. Although more detailed functional characteristics remain to be analyzed, this enzyme might

correlate with asexual and sexual development with a fundamental biological function. Therefore, the results reported here offer evidence that compounds of inhibitors could be effective candidates for novel drug discovery against chicken coccidiosis.

ACKNOWLEDGMENT. This work was partly supported by Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (grant number 22700773 to M. M.).

REFERENCES

- Dahl, E. L. and Rosenthal, P. J. 2005. Biosynthesis, localization, and processing of falcipain cysteine proteases of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **139**: 205–212. [Medline] [CrossRef]
- Geysen, J., Ausma, J. and vanden Bossche, H. 1991. Simultaneous purification of merozoites and schizonts of *Eimeria tenella* (Apicomplexa) by Percoll flotation and assessment of cell viability with a double fluorescent dye assay. *J. Parasitol.* **77**: 989–993. [Medline] [CrossRef]
- Huang, R., Que, X., Hirata, K., Brinen, L. S., Lee, J. H., Hansell, E., Engel, J., Sajid, M. and Reed, S. 2009. The cathepsin L of *Toxoplasma gondii* (TgCPL) and its endogenous macromolecular inhibitor, toxostatin. *Mol. Biochem. Parasitol.* **164**: 86–94. [Medline] [CrossRef]
- Matsubayashi, M., Hatta, T., Miyoshi, T., Anisuzzaman, Alim, M. A., Yamaji, K., Shimura, K., Isobe, T. and Tsuji, N. 2012. Synchronous development of *Eimeria tenella* in chicken caeca and utility of laser microdissection for purification of single stage schizont RNA. *Parasitology* **139**: 1553–1561. [Medline] [CrossRef]
- Mattig, F. R., Drössigk, U. and Entzeroth, R. 1993. A simple method for the purification of *Eimeria tenella* sporozoites. *Appl. Parasitol.* **34**: 139–142. [Medline]
- Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**: 1–6. [Medline] [CrossRef]
- Que, X., Ngo, H., Lawton, J., Gray, M., Liu, Q., Engel, J., Brinen, L., Ghosh, P., Joiner, K. A. and Reed, S. L. 2002. The cathepsin B of *Toxoplasma gondii*, toxopain-1, is critical for parasite invasion and rhoptry protein processing. *J. Biol. Chem.* **277**: 25791–25797. [Medline] [CrossRef]
- Rieux, A., Gras, S., Lecaille, F., Niepceron, A., Katrib, M., Smith, N. C., Lalmanach, G. and Brossier, F. 2012. Eimeripain, a cathepsin B-like cysteine protease, expressed throughout sporulation of the apicomplexan parasite *Eimeria tenella*. *PLoS One* **7**: e31914. [Medline] [CrossRef]
- Ryan, R., Shirley, M. and Tomley, F. 2000. Mapping and expression of microneme genes in *Eimeria tenella*. *Int. J. Parasitol.* **30**: 1493–1499. [Medline] [CrossRef]
- Schaeffer, M., Schroeder, J., Heckerath, A. R., Noack, S., Gassel, M., Mottram, J. C. and Selzer, P. M. 2012. Coombs GH. Identification of lead compounds targeting the cathepsin B-like enzyme of *Eimeria tenella*. *Antimicrob. Agents Chemother.* **56**: 1190–1201. [Medline] [CrossRef]
- Sijwali, P. S., Koo, J., Singh, N. and Rosenthal, P. J. 2006. Gene disruptions demonstrate independent roles for the four falcipain cysteine proteases of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **150**: 96–106. [Medline] [CrossRef]

12. Stotish, R. L., Simashkevich, P. M. and Wang, C. C. 1977. Separation of sporozoites, sporocysts, and oocysts of *Eimeria tenella* by centrifugal elutriation. *J. Parasitol.* **63**: 1124–1126. [[Medline](#)] [[CrossRef](#)]
13. Tsuji, N., Kamio, T., Isobe, T. and Fujisaki, K. 2001. Molecular characterization of a peroxiredoxin from the hard tick *Haemaphysalis longicornis*. *Insect Mol. Biol.* **10**: 121–129. [[Medline](#)]
14. Wilkins, M. R., Lindskog, I., Gasteiger, E., Bairoch, A., Sanchez, J. C., Hochstrasser, D. F. and Appel, R. D. 1997. Detailed peptide characterization using PEPTIDEMASS—a World-Wide-Web-accessible tool. *Electrophoresis* **18**: 403–408. [[Medline](#)] [[CrossRef](#)]