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Research paper

Vaccine potential of recombinant cathepsinL1G against *Fasciola gigantica* in mice

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ABSTRACT

In this study, we characterized and investigated the vaccine potential of FgCatL1G against *Fasciola gigantica* infection in mice. Recombinant mature FgCatL1G (rmFgCatL1G) was expressed in *Escherichia coli* BL21. The vaccination was performed in Imprinting Control Region (ICR) mice (n = 10) by subcutaneous injection with 50 μg of rmFgCatL1G combined with Freund's adjuvant. Two weeks after the second boost, mice were infected with 15 metacercariae by the oral route. The percents of protection of rmFgCatL1G vaccine were estimated to be 56.5% and 58.3% when compared with non vaccinated-infected and adjuvant-infected controls, respectively. Antibodies in the immune sera of vaccinated mice were shown by immunoblot to react with the native FgCatL1s in the extract of all stages of parasites and rmFgCatL1H, recombinant pro – FgCatL1 (rpFgCatL1). By immunohistochemistry, the immune sera also reacted with FgCatL1s in the caecal epithelial cells of the parasites. The levels of IgG1 and IgG2a in the immune sera, which are indicative of Th2 and Th1 immune responses, were also increased with IgG1 predominating. The levels of serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) in rmFgCatL1G-immunized group showed no significant difference from the control groups, but pathological lesions of livers in rmFgCatL1G-immunized group showed significant decrease when compared to the control groups. This study indicates that rmFgCatL1G has a vaccine potential against *F. gigantica* in mice, and this potential will be tested in larger livestock animals.

1. Introduction

Cathepsin L (CatL) is a family of proteases essential for *Fasciola* spp. survival, as it is involved in both parasite migration and digestion (Dalton and Heffernan, 1989; Collins et al., 2004). These enzymes were secreted by caecal epithelia of both juvenile and adult parasites (Sansri et al., 2013; Meemon et al., 2010). Many isotypes of CatLs have been characterized from *F. gigantica*, ie., CatL1, CatL1H, CatL1G, CatL1D, CatL2, CatL4, CatL5 (Grams et al., 2001; Norbury et al., 2011). Several forms were found for all members of CatLs, each comprising of a signal peptide, a pro-protein, a mature protein having varied sizes and molecular weights. In *Fasciola hepatica* (Fh) CatL1s are cysteine proteinases, stored as inactive forms in the zymogen granules of caecal epithelial cells (Dalton and Heffernan, 1989; Collins et al., 2004). Then the enzymes were released into the caecal lumen in the excretory-secretory (ES) materials, and the active forms were activated to cleave hemoglobin at acidic pH. In newly excysted juveniles (NEJ) the enzymes also help to break down intestinal extracellular matrix proteins during the parasite's invasion and migration through the host's tissues (Berasain et al., 1997; Lowther et al., 2009). *F. hepatica* and *F. gigantica* CatLs were closely similar in sequences, for example, FhCatL5 showed 99% amino acid identity with FgCatLIG (Norbury et al., 2011).

Various Fh proteins have been tested and shown to have varying percents of protection against *F. hepatica* infection, including leucine aminopeptidase (LAP) in sheep (Maggioli et al., 2011a), cathepsin L (CatL) in sheep and cattle (Piacenza et al., 1999; Golden et al., 2010), thioredoxin glutathione reductase (TGR) in rabbit (Maggioli et al., 2011b) and saposin-like protein 2 (SAP-2) in rabbit and mice (Espino and Hillyer, 2004; Espino et al., 2010). For *F. gigantica*, LAP have been tested in mice and buffaloes (Changklungmoa et al., 2013; Raina et al., 2011), SAP-2 in mice (Kueakhai et al., 2013a), CatL1 in mice (Kueakhai et al., 2015), CatL1H in mice (Sansri et al., 2015), CatB2 and CatB3 in mice (Chantree et al., 2013), and again they showed varying degrees of protection perhaps due to

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their immunogenicities and abundance in the parasite's ES materials.

FgCatL1G is a major CatL1 protease secreted by the NEJ and juvenile stages and it has been cloned and functionally characterized by Norbury et al. (2011). However, its tissue sources and levels of expression and in various developmental stages of *F. gigantica* and its vaccine potential has not yet been investigated. In a previous report rats vaccinated with rpFgCatL1G using Quill A adjuvant showed only 49.3% protection against *F. hepatica* (Jayaraj et al., 2009). The present study showed that FgCatL1G is a major protease produced and secreted by the caecum of NEJ and early juveniles, and it can confer a high degree of protection against *F. gigantica* infection in mice.

2. Materials and methods

2.1. Cloning and sequence analyses of cDNA of recombinant mature FgCatL1 (rmFgCatL1G)

The rmFgCatL1G was synthesized from *F. gigantica* metacercarial cDNA. The specific primers of rmFgCatL1G genes were designed from the conserved sequence of FgCatL1G gene (GenBank accession no. AF419329). The fragment of rmFgCatL1G was inserted into the pGEM-T easy vector (Promega, Madison, USA) and transformed into *Escherichia coli* DH5α. Finally, the purified plasmid-DNA isolated from a single colony of rmFgCatL1G was sequenced by Macrogen Inc (South Korea).

2.2. Expression and purification of rmFgCatL1G

The rmFgCatL1G cDNA was subcloned into the pET-30b vector and transformed into *Escherichia coli* BL21 (DE3). The protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h at 37 °C, and purified by using nickel-nitrilotriacetic acid (Ni-NTA) affinity column under denaturing condition. The elute was dialyzed by using SnakeSkin™ Pleated Dialysis tube as described by Kueakhai et al. (2013b). The rpFgCatL1 and rmFgCatL1H were expressed and purified using a method described previously (Sansri et al., 2013; Kueakhai et al., 2015). The recombinant protein was kept at −20 °C until use.

2.3. Collections of all developmental stages of parasites and preparations of parasite antigens

Lymnea ollula snails were infected with miracidia (with one miracidium per snail). About 45 days after infection, cercariae were shed from the snails and settled on pieces of 5 × 5 cm cellophane papers and transformed into metacercariae as previously described (Kueakhai et al., 2013a). The metacercariae were collected from the papers and washed several times with 0.85% NaCl solution. NEJ was excysted by activating metacercariae using a method described previously (Sethadavit et al., 2009; Kueakhai et al., 2011). The 4-week-old juveniles were collected from Golden Syrian hamsters orally infected with *F. gigantica* metacercariae. Adult parasites and eggs were collected from the bile ducts and gallbladders of naturally infected cattle killed at a local abattoir in Patumthanee province, Thailand. The whole body (WB) extracts of NEJs, juveniles and adults were prepared as previously described (Kueakhai et al., 2013a,b). For the excretory-secretory antigens (ES), the fresh 4 week-old juveniles were washed with 0.85% NaCl and incubated in RPMI medium at 37 °C, the culture medium was collected every 6 h for 24 h. After incubation, the medium was pooled and centrifuged at 5,000g at 4 °C for 20 min (Kueakhai et al., 2013a). One micro molar of phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF) was added to the supernatant and then the mixture was filtered through 0.22 μm millipore filter. The collected solution contained ES antigens whose protein concentrations were determined by Lowry's method (Bio-Rad). All samples were kept at −80 °C until use.

2.4. Experimental animals and vaccination protocol

Fifty 8-week-old male ICR mice were divided into four groups: 1) non-immunized and uninfected, 2) non-immunized and infected, 3) immunized with Freund's adjuvant (Sigma-Aldrich, St Louis, MO, USA) and infected, 4) immunized with 50 μg of rmFgCatL1G mixed with Freund's adjuvant and infected. All mice were kept under normal condition as described previously (Kueakhai et al., 2013a), and all experimental protocols were approved by The Animal Care and Use Committee, Burapha University, Chonburi, Thailand. The adjuvant control and rmFgCatL1G-vaccinated groups were immunized three times at 2-week intervals by subcutaneous route. For the rmFgCatL1G-vaccinated groups, the first immunization used the recombinant protein mixed with Freund's complete adjuvant, the first and the second boosts used the recombinant protein mixed with Freund's incomplete adjuvant. Each mouse was orally infected with 15 *F. gigantica* metacercariae at 2 weeks after the second boost. Blood samples were taken at six intervals, ie., at 0 week (prevaccination), 2 weeks, 4 weeks, 6 weeks (infection), 8 weeks, and 10 weeks (termination) post infection. Four weeks after infection, mice were anaesthetized with CO_2 inhalation and blood was taken by cardiac puncture, then their peritoneal cavities were opened and washed thoroughly with 0.85% NaCl solution. The livers were collected, immersed and minced in 0.85% NaCl solution, and the numbers of worms were examined following a previously described protocol (Kueakhai et al., 2015).

2.5. Worm recovery and worm sizes

The percent of protection against *F. gigantica* metacercaria infection in mice was expressed as percent of reduction in the number of worms being recovered from the vaccinated mice compared with non-immunized-infected or adjuvant-infected control mice, and calculated as follows:

$$\%\,\mathrm{Protection} = (\mathrm{A} - \mathrm{B})/\mathrm{A} \times 100$$

"A" represents the mean worm recovery from the non-immunized-infected or adjuvant-infected control mice, and "B" represents the mean worm recovery from rmFgCatL1G-vaccinated mice. Worm sizes (body weight) from the vaccinated mice were measured by weighing the pooled samples, then the weight of an individual worm was calculated and compared to those from the non-immunized-infected or adjuvant-infected control mice.

2.6. Determination of IgG1 and IgG2a levels by indirect ELISA

The levels of IgG1 and IgG2a in blood samples collected from six intervals as previously mentioned were determined by indirect ELISA. Ninety six-well plates were coated with 100 μl of 1 μg/ml of rmFgCatL1G in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at 4 °C, overnight (O/N). The coated plates were washed three times with 0.05% PBS-Tween 20, and the non specific binding was blocked by adding 300 μl per well of 4% skim milk (Merck KGaA, Darmstadt, Germany) in PBS and incubated at room temperature (RT) for 1 h. Then the coated plates were washed three times with 0.05% PBS-Tween 20, and 100 μl of each serum in PBS at 1:10,000 dilution was added and incubated for 2 h at RT. The plates were washed three times with 0.05% PBS-Tween 20 and incubated in HRP-conjugated goat anti-mouse IgG1 and IgG2a (SouthernBiotech, Birmingham, USA) diluted with PBS at 1:5,000 for 1 h, at RT. Then the plates were washed three times with 0.05% PBS-Tween 20,