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Schistosoma mekongi cathepsin B and its use in the development of an immunodiagnosis

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ABSTRACT

Schistosomiasis mekongi is one of the most important human parasitic diseases caused by *Schistosoma mekongi* in South-east Asia. The endemic area is the Mekong River sub-region from Laos to Cambodia. This parasite also infects dogs and pigs which are its alternative host species. Currently, the lack of reliable rapid diagnosis makes it difficult to monitor the infection and spreading of the disease. In this study, we screened the antigens of the parasite with sera of infected mice using Western blotting and identified proteins of interest with LC–MS/MS to obtain potential candidate proteins for diagnostic development. This assay yielded 2 immunoreactive bands at molecular masses of 31 and 22 kDa. The 31 kDa protein was the major band identified as cathepsin B, and its gene was cloned to obtain a full cDNA sequence (SmekCatB). The cDNA consisted of 1123 bp and its longest reading frame encoded for 342 amino acids with some putative post translation modifications. The recombinant SmekCatB (rSmekCatB) with hexahistidine tag at the C-terminus was expressed in *Escherichia coli* and purified by Ni-NTA resin under denaturing conditions. The rSmekCatB reacted with sera of *S. mekongi*-infected mice. Indirect ELISA using rSmekCatB as the antigen to detect mouse antibodies, revealed a sensitivity of 91.67% for schistosomiasis mekongi and the specificity of 100%. Our data suggested that SmekCatB is one of the most promising parasitic antigens that could be used for the diagnosis of *S. mekongi* infection.

1. Introduction

Schistosomiasis is an important global, socioeconomically devastating parasitic disease caused by *Schistosoma* species (WHO, 2006). The symptoms of the infected patients include abdominal pain, diarrhea, fever, bloody stool and finally the development of liver damage and possibly kidney failure (da Silva et al., 2013; Elbaz and Esmat, 2013). In Asia, the disease is caused by the oriental schistosomes including *Schistosoma japonicum* which is prevalent in China, the Philippines and Indonesia (Olveda et al., 2014; Satrija et al., 2015; Zhou et al., 2010), and *Schistosoma mekongi* which has been reported in the Mekong River sub-region, especially in Cambodia and Laos (Muth et al., 2010). In 2002, in Laos and Cambodia the number of people at risk from exposure to *S. mekongi* was estimated to be approximately 140,000 (Urbani et al., 2002) and continually increased to nearly 1.5 million people in 2008 (Attwood et al., 2008a,b). Moreover, domestic animals including dogs and pigs are also definitive hosts acting as reservoirs of infection (Matsumoto et al., 2002; Ohmae et al., 2004; Strandgaard et al., 2001; Urbani et al., 2002). The intermediate snail host, *Neotricular aperta*, is particularly prevalent in the areas of rocky bank along the Mekong River Basin. Three strains of *N. aperta* (γ, β, α) snails were differentiated by the appearance of pigmentation and sizes. All strains are potential intermediate hosts, but only γ-strain is the most epidemiologically significant (Attwood et al., 1997). These snails are especially abundant in Kratié and Stung-Treng Provinces in Cambodia, Khong Island in Laos, and around the juncture of the Mul and Mekong Rivers in Thailand (Attwood et al., 2008a; Sornmani,

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1976; Taguchi et al., 2000). Recently, the distribution of this intermediate snail host has been reported to be more widely spread than previously thought, and it may expand to the northern part of southern Laos, particularly in Khammouane Province (Attwood, 2001; Attwood et al., 2008b).

Diagnosis is a crucial step that must be carried out before treatment, and is also important for monitoring the spread of the disease. Epidemiological surveys, community control programs, and assessment and evaluation of control strategies are based on information gained from diagnostic results (Ross et al., 2001). The Kato-Katz detection of eggs in excreta is the gold standard, which was recommended as the method for field diagnosis by the World Health Organization (1993) (WHO). However, this method is labor-intensive, time-consuming and has limited sensitivity especially for detecting the disease in the low-prevalent endemic area and in individuals with low worm burden. In addition, it cannot be used for early detection (Lier et al., 2009; Luo et al., 2009; Utzinger et al., 2001; Yu et al., 1998; Zhang et al., 2009; Zhu, 2005). Immunodiagnosis is considered to be more sensitive than this conventional method (Alarcón de Noya et al., 2007; Cesari et al., 1987). However, several studies have demonstrated that immunodiagnosis using crude antigens from *S. japonicum* and *Schistosoma mansoni* yielded unsatisfactory and nonspecific results with the limitations of cross-reactivity with other parasitic infections and also the limitation in discrimination between active infections and past infections (Alarcon de Noya et al., 1996; Attallah et al., 1999; Boctor and Shaheen, 1986; Chand et al., 2010; Li et al., 1996, 1997; Tosswill and Ridley, 1986; Yu et al., 2011). For this reason the use of purified or recombinant antigens in immunological assays could give better sensitivity, specificity, and potentially distinguish active infection from prior infection (Doenhoff et al., 2004 Jin et al., 2010). Currently, no immunological diagnosis using specific *S. mekongi* antigen has been reported (Kirinoki et al., 2011; Nickel et al., 2015).

In this study, the screening of raw antigens of *S. mekongi* adult worms was performed by Western blot using whole fluke antigens probed by infected mouse sera. Next theimmunoreactive proteins were selected and further identified and characterized by LC–MS/MS. Our preliminary results indicated that cathepsin B (SmekCatB) was the major antigen that was detected by the mouse immune sera. The full cDNA sequence of SmekCatB gene was cloned and characterized. Then the recombinant protein (rSmekCatB) was expressed in *E. coli* and used for developing the Indirect ELISA assay which showed high specificity and sensitivity for schistosomiasis mekongi in mice.

2. Materials and methods

2.1. Collection of adult parasites

S. mekongi (Laotian strain) was maintained in *N. aperta* snails and mice (ICR strain) at the Applied Malacology Unit, Department of Social Medicine and Environment, Faculty of Tropical Medicine, Mahidol University, Bangkok. Fifty of the ICR mice were individually infected with thirty of *S. mekongi* cercariae derived from experimentally infected *N. aperta* by the looping methods (Sornmani et al., 1973). At fifty-six days post-infection, mice were sacrificed by over inhalation of CO_2 and adult flukes were collected by perfusion using 0.85% normal saline solution. After washing the flukes several times with 0.85% normal saline solution the vital flukes, as judged by the normal macroscopic appearance and active motility, were selected and stored at −80 °C until use. The protocol involved in using laboratory animals was approved by the Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University, (FTM-ACUC 006/2005).

2.2. Serum samples

The blood was drawn from normal and *S. mekongi*-infected mice at days 7, 14, 21, 28, 35, 42, 49, and 56 post-infection, respectively. All blood samples were allowed to coagulate at room temperature for 2 h and then centrifuged at 10,000 × *g* for 5 min at 4 °C to remove the coagulum. All serum samples were stored at −20 °C until use.

2.3. Preparation of adult worm antigens (AWA)

The adult *S. mekongi* were homogenized in a lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 30 mM Tris–HCl, 0.001% peptidine A and 1 mM PMSF, pH 8.8). The adult worm antigens (AWA) were then centrifuged at 12,000 × *g* for 30 min at 4 °C to remove cell debris and the protein concentrations were determined by Bradford assay. The AWA were stored at −80 °C until use.

2.4. SDS-PAGE and LC–MS/MS

The AWA were separated by SDS-PAGE in a 12.5% mini gel (6 × 9 mm) as previously described by Laemmli (1970). The AWA of *S. mekongi* were run at 25 mA in a preparative gel. Molecular weight standards were run simultaneously. After separation, the proteins were either visualized by Coomassie Brilliant Blue stain or detected by immunoblotting. Protein bands of interest were excised, digested and identified with LC–MS/MS as described previously (Abere et al., 2012; Khoontawad et al., 2012; Prajanban et al., 2012) at Proteomics Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Thailand.

2.5. Western blot analysis

The AWA of *S. mekongi* were separated by SDS-PAGE and transferred onto polyvinyldifluoride (PVDF) membrane (using 200 mA for 2 h). The membranes were immersed in a blocking solution (5% (w/v) skim milk, 2% (w/v) bovine serum albumin (BSA) in PBST (PBS containing 0.05% Tween-20) overnight at 4 °C with continuous shaking, and then incubated with either *S. mekongi*-infected mouse serum (diluted at 1:1000) as the primary antibody or non-infected mouse serum as the negative control for 2 h at room temperature. The membrane was then washed in PBST four times before being incubated further in the secondary antibody, goat anti-mouse IgG conjugated to HRP (1:5000) for 1 h at room temperature. The immunoreactive bands are detected by adding of HRP substrate using enhanced chemiluminescence kit (Pierce, USA) and detected on Hyperfilm (Kodak, Japan).

2.6. Cloning of partial SmekCatB cDNA sequence by 5′ and 3′ RACE-PCR

Total RNA was isolated from the whole adult *S. mekongi* worms by TRIzol reagent (Molecular Research Center, Inc.) using the protocol provided by the manufacturer, and stored at −80 °C. The partial cDNA sequence of the SmekCatB was amplified by reverse transcription and PCR using the following set of degenerated oligonucleotide primers: CatBFw (5′-ATGTTGAAAATCGCAGTTTGTATTG-3′) and CatBRw (5′-TTAGGTTTTTATAAGTCCAGCCACA-3′). These degenerated primers were designed from a *S. japonicum* cathepsin B cDNA sequence clone SJCHGC00054 (GenBank accession no. AY814659.1). The 5′ and 3′ nucleotide sequences of S. mekongi cDNA were amplified using 5′ RACE system for rapid amplification of cDNA ends, version 2.0 (Invitrogen) and 3′-Full RACE core set (Tagara), respectively, according to the manufacturer's instructions. The PCR was performed in 35 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s. The PCR product was