CHAPTER IV

EVALUATION OF RECOMBINANT *OPISTHORCHIS VIVERRINI* CATHEPSIN B-1 PROTEASE AS A SERODIAGNOSTIC ANTIGEN FOR HUMAN OPISTHORCHIASIS

4.1 Introduction

Opisthorchiasis remains a major public health problem in Southeast Asia, particularly in Thailand where the highest prevalence occurs in northeastern provinces including Khon Kaen, Maha Sarakham, Nong Khai and Sisaket (Sripa et al., 2010a). Although control programs have been implemented at the community level for more than three decades, prevalence remains high (Sithithaworn and Haswell-Elkins, 2003; Sripa et al., 2010a; Sripa and Pairojkul, 2008). Appropriate methods to ascertain prevalence and infection intensity are critical for designing suitable interventions and control. To date, several techniques have been developed to diagnose opisthorchiasis, in addition to parasitological methods focused on microscopical identification of *O. viverrini* eggs in stool samples. These latter techniques exhibit only low sensitivity in light infections (Haswell-Elkins et al., 1991a; Sithithaworn and Haswell-Elkins, 2003; Sithithaworn et al., 1991). Although fecal examination for the characteristically shaped eggs provides definitive diagnosis of *O. viverrini* infection, serodiagnosis for the detection of specific antibody can be expected to have advantages as an alternative diagnostic tool for opisthorchiasis when large populations in endemic regions such as the provinces of the Khorat Plateau of Thailand and the Lao provinces bordering the Mekong River, and especially for epidemiological surveys.

Detecting antibody in serum is a suitable method to estimate infection rates (Haswell-Elkins et al., 1991b). In past epidemiological surveys, crude extracts of *O. viverrini* worms – either from the tissues (somatic antigen preparations) and/or the excretory/ secretory products (ES) released by cultured flukes *in vitro* – have been employed as antigens for serodiagnosis (Tesana et al., 2007; Wongratanacheewin et al., 1988). However, preparation of these kinds of antigens is tedious with maintenance of the *O. viverrini* parasite in the laboratory and samples of

metacercariae to infected laboratory hamsters reliant on natural sources of infected, wild fishes - which is unpredictable and variable depending on the season (Wongratanacheewin, Sermswan, and Sirisinha, 2003). In addition, potential batch-to-batch variability and serodiagnostic performance compound the unsatisfactory attributes of reliance on somatic and ES antigens of *O. viverrini* for routine serodiagnosis.

By contrast, the use of a recombinant antigen for antibody detection is advantageous since recombinant proteins can be produced in preparative scales to develop more convenient and inexpensive serological assays. Recently, the performance of recombinant asparaginyl endopeptidase of *O. viverrini* was described. However, wide scale deployment of this antigen for serodiagnosis has been hampered because of difficulties in refolding the recombinant protease to a natural, soluble conformation (Laha et al., 2008). By contrast, the Sm31 antigen or cathepsin B (SmCB-1) of the human blood fluke, *Schistosoma mansoni* has been widely used for serodiagnosis of schistosomiasis mansoni (Dalton, McKerrow, and Brindley, 2004; Klinkert et al., 1991). In the present study, we produced a recombinant form of the cysteine protease cathepsin B1 of *O. viverrini* and investigated its performance and potential in an enzyme linked immunosorbent assay for serodiagnosis of human opisthorchiasis.

4.2 Materials and methods

4.2.1 Source of serum samples

A total of 145 human serum samples used for the establishment and testing of ELISA and immunoblotting assays were collected from the villages in opisthorchiasis endemic areas in Khon Kaen province, Thailand, supplied by the Tropical Disease Research Laboratory, Khon Kaen University. The samples included 87 sera from subjects with egg-positive *O. viverrini* infection and 58 sera from subjects who were negative by fecal microscopy for *O. viverrini* infection but positive for *Strongyloides stercoralis* (8), minute intestinal flukes (14), echinostomes (11), hookworms (15), *Taenia* species (3) and *Giardia lamblia* (7). Twenty sera from subjects who were negative by fecal examination and lived in non-endemic area of opisthorchiasis were used as negative control samples. In each of these 145 cases, the

infection status with a number of gastro-intestinal parasites was established by microscopic examination of stool samples prepared using the formalin ethyl acetate concentration technique (FECT) (Elkins et al., 1991). Corresponding sera were aliquoted and stored at -20 °C until used. Collection of these samples was approved by the Ethic committee of Khon Kaen University, approval no. HE451132.

4.2.2 Production of recombinant *O. viverrini* cathepsin B1

Recombinant *O. viverrini* cathepsin B1 (r*Ov*-CB-1) (GenBank accession number ACT99885) was produced in *Pichia pastoris* as described (Sripa et al., 2010b). Recombinant proteins were purified using Ni-NTA affinity columns (Novagen) and dialyzed against phosphate buffer saline (PBS) at pH 7.2-7.4 through dialysis membrane (SnakeSkin™ Pleated Dialysis Tubing, Pierce) for 4 h at 4°C and stored at -20°C until used. Protein concentration was determined by the method of Bradford (Bradford, 1976). The protein was catalytically active as described by us earlier (Sripa et al., 2010b).

4.2.3 SDS-PAGE and immunoblotting

SDS-PAGE (Laemmli, 1970) was carried out using a Mini Protein® III cell (Bio-Rad) under reducing conditions. The r*Ov*-CB-1 was dissolved at dilution 1:1 in a sample buffer (0.5 M Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue, 0.02% 2-mercaptoethanol) (100 µl, 0.5 mg/ml) and was sized-separated through 15% gel in Tris-glycine electrode buffer (25 mM Tris, 192 mM glycine, 1% SDS, pH 8.3). Following SDS-PAGE, separated proteins were transferred to nitrocellulose membrane (Bio-Rad) using a semidry blotter (Bio-Rad). The membrane was cut into strips and blocked with 5% non-fat milk powder in PBS, pH 7.4 containing 0.05% Tween 20, overnight at 4 °C. The membrane strips were incubated with human serum at a dilution of 1:100 for 2 h and then washed with PBST, five times, each wash for 5 min. The strips were incubated for 2 h with goat anti-human IgG conjugated with horseradish peroxidase (Zymed) at dilution 1:1000. Subsequently, strips were washed with PBST, four times, 5 min and final wash with PBS after which immunoreactivity was visualized with the substrate *o*-diaminobenzidine (DAB) (Zymed).

4.2.4 Enzyme linked immunosorbent assay (ELISA)

r*Ov*-CB-1 in PBS was diluted to 0.5 μg/ml in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6 (coating buffer) and applied to a 96 well-plate (Nunc Maxi-Sorp Immuno Plate, Denmark), 100 μl/well, overnight at 4°C. Each well was washed with 200 μl of 154 mM NaCl, 0.05% Tween-20 (wash buffer) for 3 min, three times. Antigen was blocked with 200 μl/well of 3% BSA in coating buffer at 37 °C for 2 h. Plates were washed with 200 μl/well of wash buffer for 3 min, three times. One hundred μl of serum diluted 1:1,000 in 3% BSA in 137 mM NaCl, 9 mM Na_2HPO_4 .$2H_2O$, 0.8 mM $NaH_2PO_4.2H_2O$, 0.05 % Tween-20 (incubation buffer) was added and incubated at 4°C overnight. Plates were washed with wash buffer, as above. The secondary antibody, horse radish peroxidase (HRP) conjugated anti-human IgG (Fc) (Zymed), was diluted 1:30,000 and 100 μl was added and incubated for 1 hour at 37°C. Plates were washed with wash buffer as above followed by an additional was with PBS for 3 min. Tablets of substrate *o*-phenylenediamine (OPD) (Zymed) dissolved in citrate buffer pH 5.0 (one tablet/12 ml) were used to develop colorimetric changes by adding 100 μl/well OPD solution and incubating for 30 minutes at 37°C. Reactions were terminated by addition of 0.5 M sulfuric acid (H_2SO_4), 100μl/well. Optical density at 492 nm (OD_{492}) was determined using a plate reader (TECAN). All samples were assayed in duplicate. Each plate was standardized with blank (non-coated antigen), positive and negative control sera. Positive control was pool positive sera from subjects who were positive *O. viverrini* egg in feces and has high IgG level against ES antigen and negative control is sera from subjects who were negative by fecal examination and lived in non-endemic area of opisthorchiasis.

4.2.5 Statistical analysis

The optimal cut-off value for ELISA was evaluated base on receiver operating characteristic (ROC) curve analysis that correlated with true and false positive rates [sensitivity and (1-specificity)] (Bon et al., 2010). ROC curve and area under the curve (AUC) were carried out using MedCalc software (http://www.medcalc.be) (Mariakerke). The sensitivity, specificity and positive and negative predictive values were calculated using the formalin-ethyl acetate concentration technique (FECT) as the gold standard method (Elkins et al., 1991).

The quantitative variables were individual test for normality with one-sample Kolmogorov-Smirnov test. The statistical significance between the different groups was performed with one-way ANOVA. Analysis of the relationship between OD_{492} and *O. viverrini* eggs per gram of human feces (EPG) was performed with the Krustal-Wallis H test (nonparametric analysis of variance). The data were analyzed using SPSS 16.0 for Windows. *P* values of ≤ 0.05 were considered to be statistically significant.

4.3 Results

4.3.1 Sera from opisthorchiasis subjects recognized recombinant *Ov*-CB-1

r*Ov*-CB-1 was expressed and secreted in soluble form into culture medium by *P. pastoris*. Affinity purified r*Ov*-CB-1 migrated in SDS-PAGE as a single species at ~44 kDa, which is the active form of the enzyme (Sripa et al., 2010b) (Figure 4.1, lane 3). Immunoblot analysis showed that r*Ov*-CB-1 was recognized by *O. viverrini* positive human sera, revealing a major band of recognition at 44 kDa, whereas control, non-*O. viverrini* infected sera showed no reactivity (Figure 4.1).

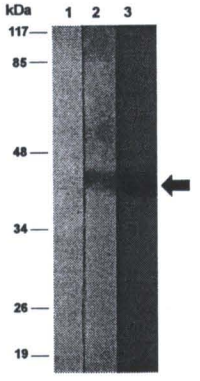

Figure 4.1 Human opisthorchiasis sera react with recombinant *Ov*-CB-1. Western blot analysis of a pool of positive human sera from individuals with parasitologically proven *Opisthorchis viverrini* infection. Lane 1, 1:100 dilution of pooled negative human sera; lane 2, 1:100 dilution of pooled positive human opisthorchiasis sera, showing recognition of r*Ov*-CB-1 at 44 kDa; lane 3, purified r*Ov*-CB-1 analyzed by Coomassie blue-stained 12.5% SDS-PAGE.

4.3.2 Efficacy of r*Ov*-CB-1 as an immunodiagnostic tool

An indirect ELISA was developed to test 145 human sera collected from an endemic area for opisthorchiasis in Khon Kaen province, Thailand. There were 87 positive *O. viverrini* infection cases, 44 male and 43 female individuals. The *O. viverrini* epg range for these 87 cases was 8 to 6,668 epg, determined using a modified formalin ethyl acetate concentration technique. The subjects of positive *O. viverrini* egg were classified into 4 groups based on range of epg. The number of subjects in each group were epg 1-500 = 63, epg 501-1000 = 11, epg 1001-1500 = 4 and epg >1500 = 9 (as shown in figure 4.2). The cut-off OD_{492} level for the positive *O. viverrini* infection case of the ELISA test calculated from ROC curve was 0.76 (Figure 4.2A). Human sera from persons diagnosed parasitologically with *O. viverrini* infection gave strong OD values, with 67% sensitivity. Sera from humans infected with other gastro-intestinal parasites showed a specificity of 82.76 % (Table 4.1). The positive and negative predictive values of the test were 85.29% and 62.34% respectively.

The correlation of IgG level and fecal epg counts among several infection intensity categories indicated that the IgG titers of the high intensity group were higher than the low intensity group (Figure 4.2B). Accordingly, r*Ov*-CB-1 exhibits potential as a diagnostic antigen for ELISA-based serodiagnosis for opisthorchiasis in this endemic area of Thailand as indicated by the concordance of absorbance value with the intensity of *O. viverrini* infection.

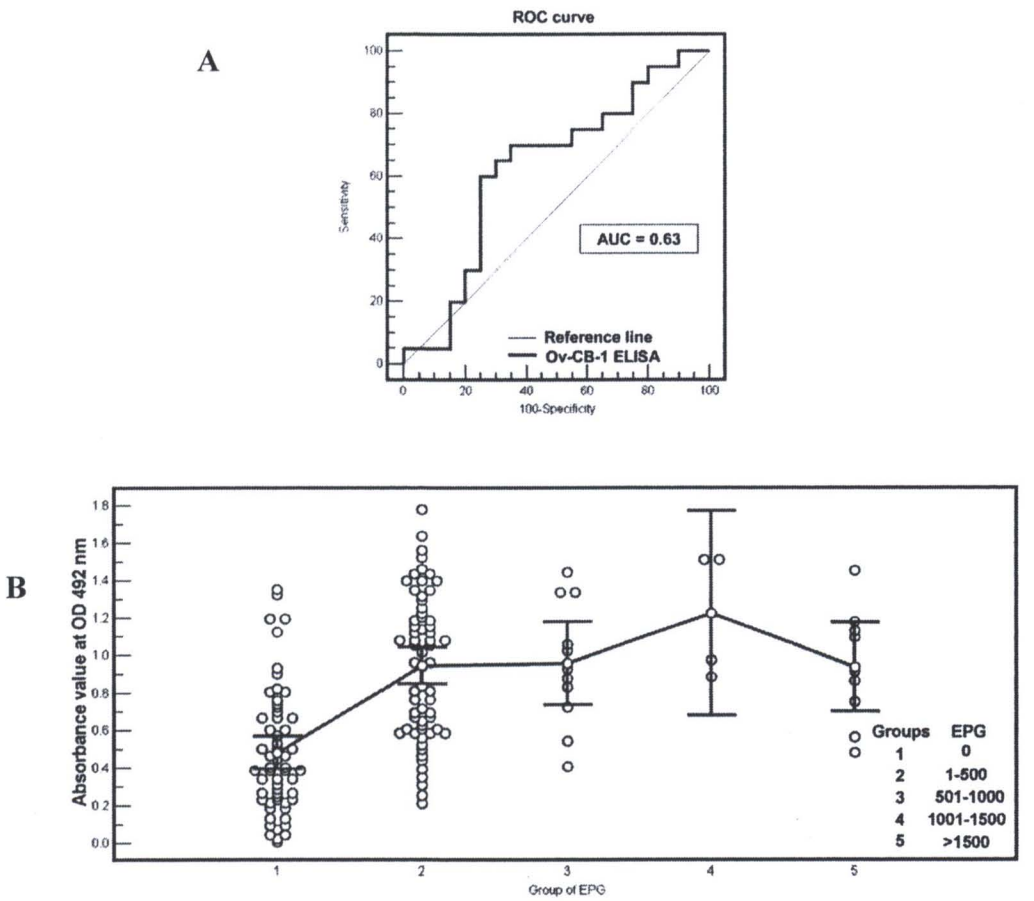

Figure 4.2 Performance of recombinant *Ov*-CB-1 as a serodiagnostic antigen. Panel A: Receiver operating characteristic (ROC) curve of recombinant cathepsin B from *Opisthorchis viverrini* (*Ov*-CB-1) employed as the antigen in an indirect ELISA, calculated from optical density (OD) at 492 nm values for sera from parasitologically negative and positive opisthorchiasis-positive and negative individuals. The curve was plotted between true positivity rate (sensitivity) and false positivity rate (100-specificity) for different cut-off points of OD. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. The curve represents the suitable cut-off point at OD 0.7637 and showed that the area under the curve (AUC) was 0.63 (95% confidence interval 0.463 to 0.777). Panel B: Serodiagnosis of *O. viverrini* infection among different infection intensity groups using the *Ov*-CB-1 ELISA.

Table 4.1 Comparison of the *Ov*-CB-1 ELISA with fecal examination using formalin-ethyl acetate concentration technique (FECT) as the gold standard method for diagnosis of opisthorchisis in 145 human sera (Panel A). With reference to FECT, *Ov*-CB-1 ELISA test shown 66.67% sensitivity and 82.76% specificity. The positive and negative predictive values were 85.29% and 62.34%, respectively. Number of the sera from other-helmintic infection subjects used for cross-reactivity assay (Panel B).

A.

Ov-CB-1 ELISA test result	No. (%) of samples from FECT method		
	Positive	Negative	Total
Positive	58	10	68 (85.29)
Negative	29	48	77 (62.34)
Total	87 (66.67)	58 (82.76)	145

B.

Parasites	No. of serum samples	No. positive of *Ov*-CB-1 ELISA (%)
Echinostome	11	2 (3.45)
Giardia lamblia	7	2 (3.45)
Hookworm	15	2 (3.45)
Minute intestinal fluke	14	2 (3.45)
Strongyloides stercoralis	8	1 (1.72)
Taenia sp.	3	1 (1.72)
Total	58	10 (17.24)

4.4 Discussion

The findings from this present study indicate that *Ov*-CB-1 shows potential for development and deployment as an immunodiagnostic tool for opisthorchiasis. Human sera from parasitologically proven cases of *O. viverrini* infection showed strong reactivity with r*Ov*-CB-1. An indirect ELISA for detection of serum IgG antibodies specific for r*Ov*-CB-1 yielded levels of sensitivity (67%) and specificity (81%). The relative ease and modest expense of production of r*Ov*-CB-1 in *P. pastoris* and affinity purification of a soluble form of this enzyme are key attributes that make r*Ov*-CB-1 particularly attractive as a serodiagnostic antigen (Sripa et al., 2010b). By way of comparison to similar antigens, the diagnostic efficiency of r*Ov*-CB-1 is similar to that exhibited by Sm31 for serodiagnosis of human schistosomiasis mansoni (El-Sayed et al., 1998; Klinkert et al., 1991). Nonetheless, there is the possibility that cross reactivity with antigens from other helminths may occur, and/or that past infection with *O. viverrini* may complicate the interpretation of positive ELISA outcomes.

An important approach will now be to determine whether *Ov*-CB-1 can differentiate between present and past infection. Fortunately, we have potential access to informative serum samples from individuals both before and following treatment with praziquantel for *O. viverrini* infection, through a large longitudinal study we are undertaking on liver fluke-induced cholangiocarcinoma in Khon Kaen province (Sripa et al., 2009). Moreover, we plan to investigate whether synthetic peptides from *O. viverrini* cathepsin B might improve sensitivity and specificity, as has been seen with Sm31 in regions of low prevalence (Noya et al., 2002).

Finally, proteases including cathepsin B, are often highly upregulated in cancer (Cudic and Fields, 2009). *Ov*-CB-1 plays a role at the host-parasite interface by degrading host tissues and establishing a pro-inflammatory environment (Sripa et al., 2010b), and might contribute to the establishment of a tumorigenic microenvironment within the infected bile ducts (Pinlaor et al., 2009; Sripa et al., 2010b).