CHAPTER V

EVALUATION OF RECOMBINANT CATHEPSIN B-1 AS VACCINE ANTIGEN AGAINST *OPISTHORCHIS VIVERRINI* INFECTION AND EFFECT ON HUMAN CANCER CELL PROLIFERATION

5.1 Introduction

Opisthorchiasis and cholangiocarcinoma that caused by *Opisthorchis viverrini* are still the problem in Southeast Asians countries, especially in Northeast of Thailand (Sripa et al., 2007). The extensive strategies have been performing to control helminth infection by using anti-helminthic drugs in the aspect of eliminate human host reservoir although drug resistance parasites may occur (van den Enden, 2009). To avoid the failure and adverse effect from drug parasite treatment, the vaccine researches are developing to prevent these problems.

To produce host immune responses that involve in worm elimination, prolong and strong host immune responses against helminth are needed to induce during the course of infection. In *O. viverrini*, protective immunity after reinfection prior to immunization has been reported in a hamster model (Sirisinha et al., 1983a; 1983b). Protective responses have been shown in hamsters at 30% worm reduction after infected with a small number of flukes prior to immunization with aqueous somatic extract of adult worms. It was suggested that induction of protective immunity in *O. viverrini* infection due to immunosuppression and parasite can evade immune system of the host (Wongratanacheewin et al., 2003). In the related fluke *Clonorchis sinensis*, several antigens have been evaluated as vaccine candidates; a DNA vaccine for cathepsin L gave 32% protection in rats (Lee et al., 2006) and recombinant tegumental protein 22.3 kDa (CsTP22.3), 42% protection (Zhou et al., 2008). Irradiated MCs provide significant protection in rats against *C. sinensis* infection (Quan et al., 2005). To date, the recombinant technologies are allowed to produce the specific antigen for vaccination. The recombinant vaccine seem to be more safe, cheap, stable, easy to administer, prefer single-dose administer and induce broad immune response with life-long memory than native materials.

Cathepsin B-like proteases have been proposed as the mediator for parasitism with the important role in biological function of parasite including feeding, tissue invasion and immune evasion. Moreover, in the sense of immunogenicity, cathepsin B chiefly comprises in ES products of parasites and has a greater chance to expose to the immune system of the host and considered as the vaccine target. Cathepsin B has been evaluated for anti-fluke vaccine in many orgainisms such as *Fasciola hepatica* and *Schistosoma japonicum* by using single and combine with other molecules. (Jayaraj et al., 2009; Chen et al., 2005).

In *O. viverrini*, cathepsin B was found in *O. viverrini* transcript and abundantly expressed in *O. viverrini* and also detected in ES products of parasites. *O. viverrini* cathepsin B have a great potential to interact with or at least exposed to host tissues and host immune response (Laha et al., 2007). In addition, *O. viverrini* cathepsin B is performing important role for parasite living such as feeding and tissue invasion that cause harmful effect to host (Sripa et al., 2010b). To block the function of cathepsin B could be the effective way to inhibit the development of helminth and control *O. viverrini* infection.

In this study, we assess vaccine efficacy against *O. viverrini* infection by using cathepsin B-like cysteine proteases (*Ov*-CB-1) (Sripa et al., 2010b) as the immunogen in hamster model. The protective efficacy of this vaccine was evaluated in terms of parasitological parameters including worm burden and egg production.

As cathepsin B is one of the major digestive enzymes of this liver fluke, and we recently reported that it performs an essential function in trans-activation of the related cathepsin F protease to enhance the ability of these hydrolases to digest host tissues that the fluke ingests. In addition, it has been suggested that cathepsin B may further contribute to pathogenesis of *O. viverrini* infection by acting as an irritant when release by the flukes during their activities within the biliary tree (Sripa et al., 2010b). Accordingly, we have begun to investigate the relationship of this fluke enzyme to the tumorigenic environment of the infected biliary system, and here investigated the ability of the protease to stimulate a cell line originating from a human cholangiocarcinoma.

5.2 Materials and methods

5.2.1 Evaluation of recombinant *O. viverrini* cathepsin B as a vaccine against *O. viverrini* infection in hamster

5.2.1.1 Animals

Male Syrian golden hamsters, aged 6-8 weeks obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University were used in this experiment. The hamsters were divided into 3 groups (3 per group) for used in vaccine trial.

5.2.1.2 Antigen preparation

O. viverrini crude metacercarial extracts and purified recombinant *O. viverrini* cathepsin B-1 (r*Ov*-CB-1) were prepared and used to vaccinated hamsters. Purified r*Ov*-CB-1 was produced as previously described (Sripa et al., 2010b). To prepare *O. viverrini* crude metacercarial extracts, the *O. viverrini* metacercariae were collected from cyprinoid fish that obtained from fresh water reservoir in endemic area of Khon Kaen province. The *O. viverrini* metacercariae in fish were obtained by digestion method, in brief, fishes were minced with electric grinder prior to incubate in 0.7 N HCl-pepsin solution at 37 °C, 2 h for digest fish tissue. After that, the digest was serially filtered and washed several times with normal saline (Srisawangwong, Sithithaworn, and Tesana, 1997). Metacercariae of *O. viverrini* were identified and collected under dissecting microscope. About 1,000 metacercariae were resuspended in PBS pH 7.4 and sonicated at amplitude 25% output for 5 min at 4 °C. The suspension was determined the protein concentration by using spectrophotometer (ND-1000, NanoDrop Technologies) then dispensed in small aliquots and kept frozen at -20 °C.

5.2.1.3 Vaccination

The hamsters were divided into 3 experiment groups including vaccinated with recombinant *Ov*-CB-1, *O. viverrini* crude metacercarial extracts and deionized (DI) distrilled water as the negative control group (n=3/group). Each group was immunized as the details in table 5.1. In brief, single dose of 100 ug in 100 ul of r*Ov*-CB-1 or crude metacercarial extract were injected subcutaneously to hamsters in 2 weeks interval. The antigens were formulated with 100 ul of Freund's complete adjuvant (Sigma) for first injection and repeated in the equal volume by formulated with Freund's incomplete adjuvant (Sigma) as a booster. In the control group that given with DI water was followed with the same immunization schedule. One week after second immunization, the hamsters were challenged with 50 metacercariae orally.

5.2.1.4 Determination of worm egg counts and worm recoveries

At 1 month after challenged, the hamsters were separated to one per cage for feces collection. The feces were collected a week interval for 6 weeks to determined the number of *O. viverrini* egg per gram (EPG) with the quantitative formalin/ethyl acetate concentration technique (Elkins, Haswell-Elkins, and Anderson, 1986). The worm burdens were determined at the day of sacrificed the hamster by counting worms in the liver squashes. To determine the egg production per worm, 10 adult worms from each vaccinated group were collected and washed with physiological saline. The worms were homogenized and the numbers of eggs in the uterus were counted under the microscope.

5.2.1.5 Detection of hamster antibody response by ELISA

Pre and post-immunization sera were collected by phlebotomy of the retro-orbital venous plexus on the day before the first immunization and on the day before challenge. At the day of sacrificed hamsters, sera were bled from the heart. Sera were kept at -20 °C for antibody assay. The antibodies were determined by ELISA.

Pre and post immunization sera from hamsters were tested individually by enzyme-linked immunosorbent assay for the presence of anti-*O. viverrini* cathepsin B1 protein and crude metacercarial extracts antibodies in hamster group 1, 2 and 3. The ELISA titer was determined against *O. viverrini* cathepsin B1 protein and crude metacercarial extracts. Briefly, 0.5 ug/ml of cathepsin B1 protein and crude metacercarial extracts were coated on 96 well-plate (Nunc Maxi-Sorp Immuno Plate, Roskide, Denmark) and incubated for overnight at 4 °C. Plates were washed and antigen was blocked with 5% skimmilk and incubated at 37 °C for 2 h. One hundred ul of 1:800 hamster serum was added and incubated at 37 °C for 2 h. Plate was washed again and then HRP conjugated anti-hamster IgG (Zymed) at dilution 1:20,000 were added and for 1 h at 37°C. Plate was washed again and freshly prepared OPD substrate solution (1 tablet Zymed in 12 ml of Citrate-phosphate buffer, pH 5.0) was added and incubated for 30 minutes at 37°C. The reaction was stopped by adding 0.5 M sulfuric acid (H_2SO_4) 100µl/well. The ODs were measure on an ELISA reader (TECAN, Austria) at OD 492 nm. Each sample was always assays in duplicate. Each plate included blank (non-coated antigen).

5.2.1.6 Statistic analysis

The worm burden in each group was counted and subjected to a two-way analysis of variance to examine the effects of different vaccine antigen. The EPG and egg per worm data were subjected to a two-way analysis of variance to examine the effects of different vaccines, the week of collection (weeks 1-6), and the interactions between the different vaccines and the week of collection. All statistic analysis was analyzed using SPSS package.

5.2.2 Human cancer cell lines and cell proliferation assay

The human cholangiocarcinoma (CCA) cell line, KKU-M156 (moderately-differentiated adenocarcinoma) was maintained as previous described (Sripa et al., 2005). Briefly, cells were cultured in complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL), 100 U/ml penicillin, 100 µg/ml streptomycin) and incubated at 37 °C under 5% CO_2. To assess the effect of r*Ov*-CB-1 on CCA cell growth, a variety of co-culture were investigated. The KKU-M156 was co-cultured with r*Ov*-CB-1, excretory-secretory and somatic antigen of *O. viverrini* in 96 well-plates. In co-culture, the cells were seeded at a density of 5,000, 10,000, 15,000 and 20,000 cells/ml in complete medium and incubated in 5% CO_2 incubator at 37 °C for 24 h for cell attachment. Each well were washed with PBS and incubated in RPMI 1640 containing 1% FBS to starve the cells. *Ov*-CB-1, crude somatic extracts and excretory-secretory products at concentration 1, 5 and 10 µg/ml were added into treated cell culture and cell proliferation assay was determined at 24, 48 and 72 h with a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sripa B. unpublished; Mossman, 1983). In brief, 10 µl of 10 mg/ml MTT was added to each well then incubated the reaction mixture at 37 °C for 3 h. Purple crystals solubilized with 200 µl DMSO and quantified the formazan product by measuring at the absorbance at 590 nm on a plate reader (VersaMax, Molecular Diagnostics). To assess cell proliferation, the experiment was performed in triplicate and mean ± SD of OD590 were calculated.

The ability of each antigen to stimulate cell proliferation was determined at each time point compared with the viability of the control cells. Statistical evaluation was performed by one-way ANOVA in SPSS package. $P \leq 0.05$ was considered statistically significant.

5.3 Results

5.3.1 Evaluation of recombinant *O. viverrini* cathepsin B as a vaccine against *O. viverrini* infection in hamster

5.3.1.1 IgG levels against r*Ov*-CB-1 and crude metacercarial extracts in hamster

Serum samples from hamsters were tested for the presence of the IgG level against r*Ov*-CB-1 and metacercarial extract by using ELISA (Figure 5.1). The individual serum from pre-, post-immunization and after sacrificed hamsters were tested and calculated for mean OD ± SD at 492 nm.

In the group of vaccination with r*Ov*-CB-1, the level of IgG against r*Ov*-CB-1 increased rapidly after vaccinations and was declined after second vaccination (Figure 5.1A) but the antibody level was sustained more than two fold higher than metacercarial vaccinated and control groups. The humoral response was maintained over the time course of the duration of the experiment. The level of IgG in the group of vaccination with metacercarial extracts showed high sustainable level after vaccination but still lower than immunized with r*Ov*-CB-1 (Figure 5.1B). Moreover, IgG against metacercaria antigen was also detected in hamsters that vaccinated with *Ov*-CB-1 recombinant protein. The IgG level of pre immunized sera and sera from negative control group (immunized with DI water) were not raised against *Ov*-CB-1 protein and crude metacercarial extract.

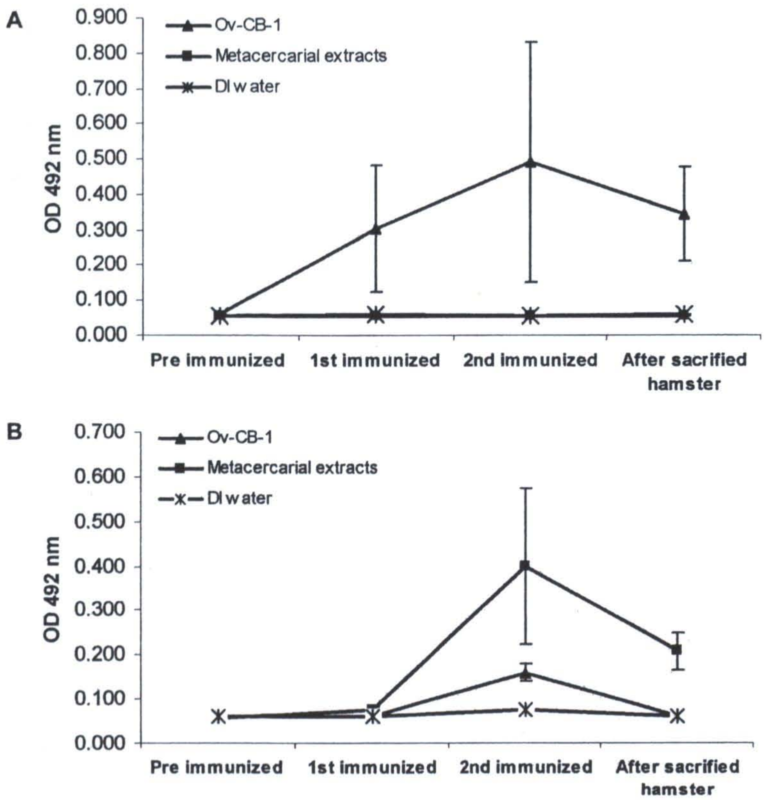

Figure 5.1 IgG level against r*Ov*-CB-1 (A) and metacercarial extract (B) in hamster after vaccination with different antigens. Hamsters were divided into 3 groups, in each group (n=3). The first group was injected with 100 ug of *Ov*-CB-1, the second group was immunized with *O. viverrini* crude metacercarial extract and the latter was injected with DI water in presence of Freund's complete adjuvant in first immunization and Freund's incomplete adjuvant in second immunization. Sera were collected before each immunization and 6 weeks post-immunization then tested individually for IgG level against *Ov*-CB-1 and metacercarial extract with ELISA coated with 0.5 ug/ml of recombinant *Ov*-CB-1 protein and *O. viverrini* crude metacercarial extract.

5.3.1.2 Reduction of worm burden and egg production in vaccination hamster

The protection against challenge infection was determined by egg production and worm burden. For egg production, feacal egg count and egg *in uterus* per worm was determined after challenge infection for one month. The result of egg production was present as egg per gram (EPG) of feces material. The EPG of vaccinated hamsters was gradually increased from week 1 to week 6 of feacal collection schedule. The variation of egg output was observed in all three groups of hamster. Although, *Ov*-CB-1 and metacercarial extract were induced significant specific antibody as detected by ELISA but EPG of those group were not reduced when compared with control group. The difference of mean egg counts between *Ov*-CB-1, metacercarial and DI vaccinated group was not significant (P>0.05) (Figure 5.2). The average worm burden was determined at the day of sacrificed and compared with control group. The significant of worm reduction was observed in metacercarial vaccinated group by presented 21.5% reduction when compared with the control group (P<0.05). There was not significant of worm reduction in *Ov*-CB-1 vaccinated group by reduce the worm to 18.08% (P>0.05). The egg production per worm was observed in all three groups of vaccination. Worms from hamsters that vaccinated with *Ov*-CB-1 and metacercarial extract were present high rate of egg production with 3,101.18 and 3,002.74 egg/worm, respectively. In contrast with control group that presented lower number of egg production than vaccinated group with 2,528 egg/worm. However, there was not different of egg production per worm among three group (P>0.05) (Table 5.1).

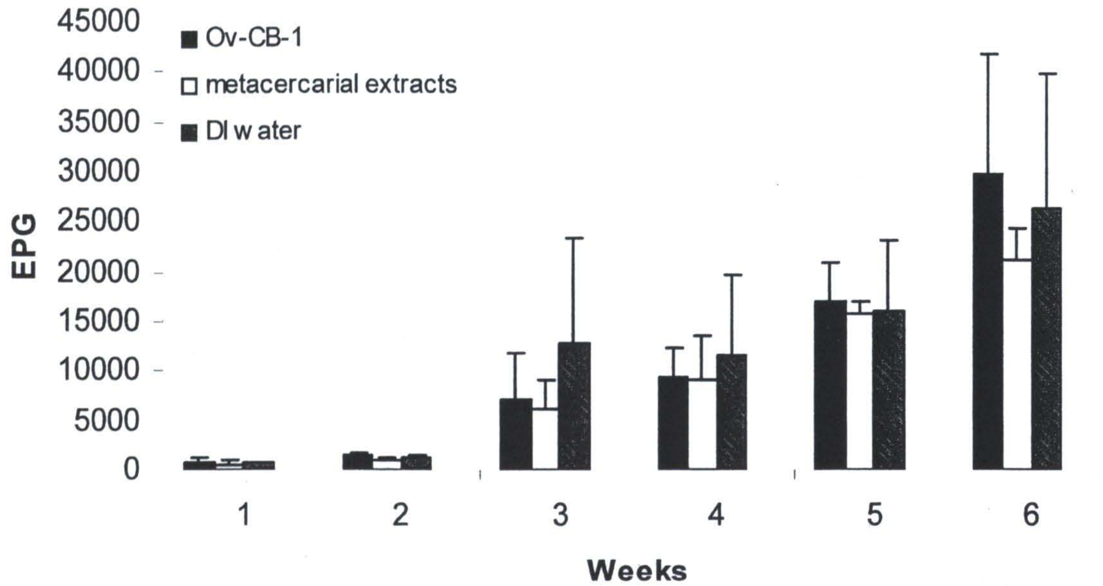

Figure 5.2 Quantitative fecal egg counts of feces from experimental hamsters that vaccination with r*Ov*-CB-1, metacercarial and DI water. The number of egg count in feces was determined as weekly interval after one month of *O. viverrini* challenged infection with 50 metacercariae. No significant difference was found among number of EPG in three vaccinated group (P>0.05). The data of EPG was presented as mean ± SD.

Table 5.1 Details of vaccination and protection against *O. viverrini* in hamsters vaccinated with *Ov*-CB-1, metacercarial extract and DI water.

Group No. 3 Hamsters/group	Antigen	Route	Dose	Adjuvant	Mean worm burdens ± SD	%reduction in worm burdens (*P*-value)	EPG/worm (*P*-value)
1	Ov-CB-1	Subcutaneously	100 ug	Freund's	24 ± 4.58	18.08 (> 0.05)	3101.18 (> 0.05)
2	Metacercarial extract	Subcutaneously	100 ug	Freund's	23 ± 1.73	21.5 (< 0.05)	3002.74 (> 0.05)
3	DI water	Subcutaneously	-	Freund's	29.33 ± 2.08	-	2528 (> 0.05)

5.3.2 Influence of liver fluke cathepsin B in proliferation of a human cholangiocarcinoma cell line

To investigate the potential activity of the *O. viverrini* cathepsin B in pathogenesis of *O. viverrini* infection and in cholangiocarcinogenesis, the human CCA cell line, KKU-M156 was co-cultured with dilutions of r*Ov*-CB-1 and with *O. viverrini* ES and somatic antigen preparations. Three concentrations of r*Ov*-CB-1 at 1, 5 and 10 μg/ml were used to investigate the role of *Ov*-CB-1 on proliferation of human CCA cell line.

After 24 h, co-cultures of the KKU-M156 cells with 10 μg/ml of r*Ov*-CB-1 showed significantly ($P \leq 0.05$) more growth than control cell cultures whereas no differences were seen between cultures exposed to OvSomatic or OvES at any protein concentration. Also, at day 3, co-cultures with 5 μg/ml of r*Ov*-CB-1 showed significantly more growth ($P \leq 0.05$) than controls whereas no differences were seen between cultures exposed to OvSomatic or OvES at any protein concentration (Figure 5.3).

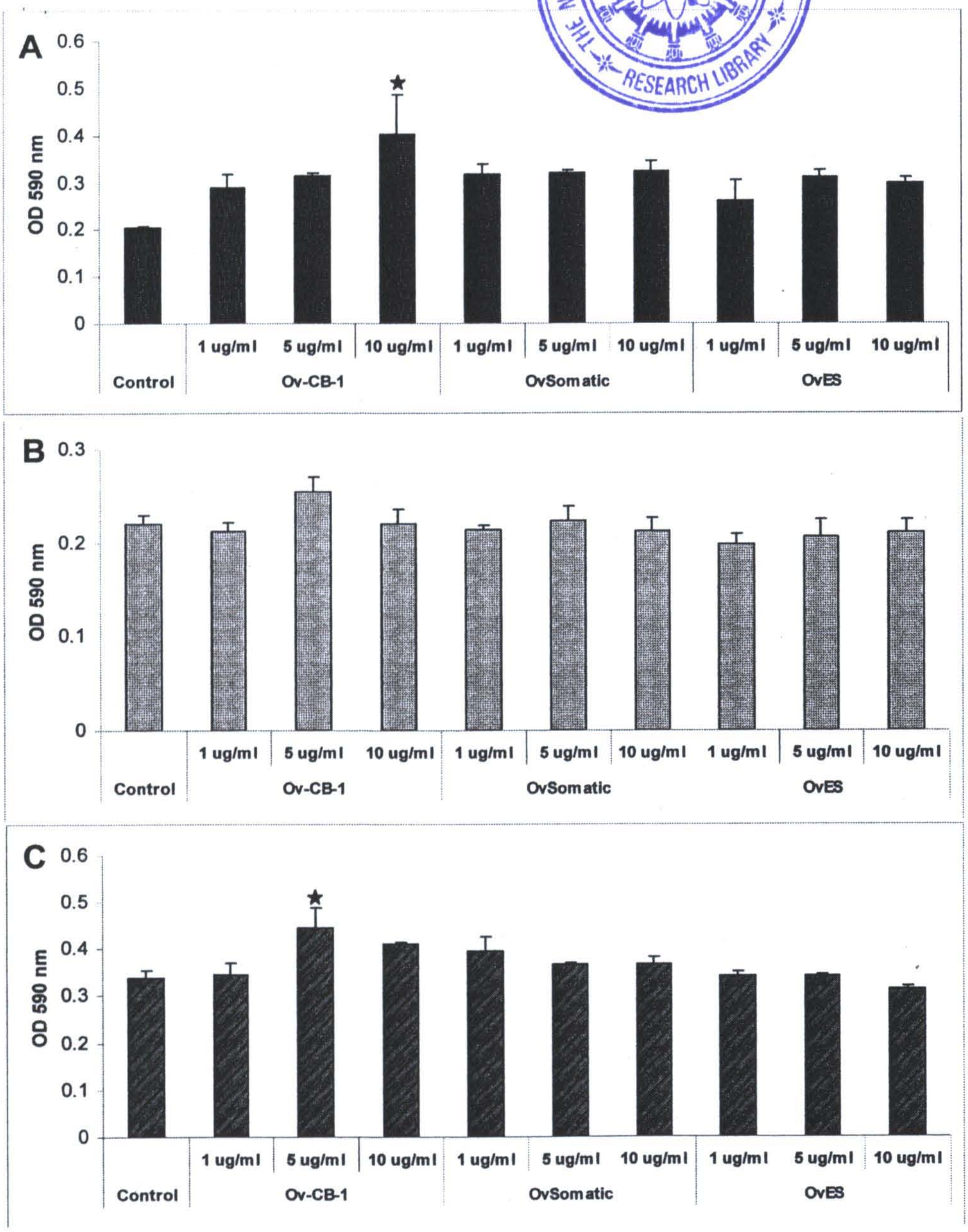

Figure 5.3 Effect of *Ov*-CB-1, *O. viverrini* crude somatic extract (OvSomatic) and excretory-secretory product (*Ov*ES) at 1, 5 and 10 ug/ml on KKU-M156 proliferation. Cell proliferation was measured by MTT assay. Data are listed as mean ± SD. The co-cultures were maintained for 3 days in 2% FBS RPMI. In day 1 (A), OD was showed in cell line that co-culture with 10 ug/ml of *Ov*-CB-1 was significantly highly than in control. There was no significant proliferation of cell line in day 2 (B) but proliferated cell was significantly higher in day 3 at 5 ug/ml of *Ov*-CB-1 (C) when compared to control.

5.4 Discussion

O. viverrini cathepsin B-like cysteine proteases are abundantly express proteases to perform important role in biological functions of helminth to complete their life cycle including feeding and tissue invasion (Sripa et al., 2010b). Cathepsin B is mediator for parasitism and considered the target for vaccination in many parasitic organisms in the aim of control parasitic infection.

In this study, a single molecule of recombinant *Ov*-CB-1 protein (r*Ov*-CB-1) was produced as active form in yeast system (Sripa et al., 2010b) and used as vaccine against *O. viverrini* infection in hamsters. r*Ov*-CB-1 is highly immunogenicity with such strong humoral response over the course of infection but lack of protection against *O. viverrini* infection. The r*Ov*-CB-1 vaccinated group was presented non-significant reduction of worm burden when compared with unvaccinated group. Meanwhile, metacercarial vaccinated group was present higher worm reduction rate than r*Ov*-CB-1 vaccinated group. This indicated that multi-antigenic molecule in metacercarial extracts present the good level of protection than single molecule of recombinant protein, although, it may not be desirable from adverse effect of the excess antibodies in circulation. Besides this vaccination trial was not showed the different of worm egg production among three groups of hamster. From these can indicated that the inducible immune responses do not affect on reproductive system of the parasites.

Due to parasites are complexity organisms, hybrid proteins or multivalent vaccines of several candidate antigens that derived from different stages of the parasite's life cycle or different species of the parasite would be more efficient vaccine in eliciting a strong immune response in the host and those are attractive to evaluate for vaccine against *O. viverrini* infection. A number of studies have demonstrated the high efficiency of hybrid or multivalent recombinant protein vaccination in infectious disease such as fascioliasis (Jayaraj et al., 2009), schistosomiasis (Chen et al., 2005), malaria (Li et al., 1999), and leishmaniasis (Coler et al., 2002). In fascioliasis cathepsin B exhibit the maximal protection against fascioliasis in mice when combine with cathepsin L by reduce worm burden, worm size and liver damage (Jayaraj et al., 2009). In the combination of cathepsin B DNA vaccine with recombinant murine IL4 can also show protective effect in mice against

Schistosoma japonicum by yield 43.2% and 76.6% reduction of worm burden and fecundity rate, respectively (Chen et al., 2005).

Moreover, helminths are long-lived organisms in specific host because they use antigenic variation to escape from the hosts' immune attack. In addition they are developed different strategies for survival in their human host. For example schistosomula can compromise complement function (Ouaissi et al., 1981) and degrade host immunoglobulins (Auriault et al., 1981). Thus, host immune response is not enough to eradicate the parasite. Vaccination is the method to increase host immunity to destroy and eradicate parasite from their host. The highly induction of specific host immune response against the parasite is important for worm elimination. Helminth infections are typically associated with hypereosinophilia, considerable IgE production, mucous mastocytosis, and goblet cells hyperplasia. The vaccinations that can induce these mechanisms may have potential to reduce parasitic infection because these immune parameters are involved in different effector mechanism to worm elimination. Moreover, the combination of the function of humoral and cell mediated host immune response may also increase the reduction of worm and decrease worm egg production (MacDonald, Araujo, and Pearce, 2002). The appropriate induction of immune response mechanisms may allow the rational development of more efficacious *O. viverrivi* vaccine.

ES from *O. viverrini* exhibits activity that stimulates host cell proliferation that likely play a pivotal role in tumorigenesis of liver fluke induced bile duct cancer (Smout et al., 2009; Sripa, 2003; Thuwajit et al., 2004). The studies presented here with the CCA cell line KKU-M156 were designed to investigate mitogenic or proliferative activity of r*Ov*-CB-1, in comparison to crude somatic and ES antigens preparations of *O. viverrini*. These findings revealed that *O. viverrini* cathepsin B could stimulate growth of this cell line, at concentrations in the range of 5 -10 μg/ml. It will be important to follow up these findings with more detailed analysis including studies with non-CCA cells such as the H69 cholangiocyte and into the interaction of the protease with Toll-like and other surface receptors (Ninlawan et al., 2011).