Gene expression in *Clonorchis sinensis* and development of DNA vaccine against clonorchiasis

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This certifies that the Doctoral Dissertation of Ji-Sook Lee is approved.

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The Graduate School

Yonsei University

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If you have great talent, industry will improve them: if you have but moderate abilities, industry will supply their deficiency

- Joshua Reynold -

All began with a strange but plausible idea, sleepless nights and days with worries followed to prove the idea. Finally, I am unspeakably great that I could collect some pieces of evidences which are presented here.

I give special thanks to my professor, Tai-Soon Yong, who has guided me through the entire process of this experiment, and I want to express my appreciation to Prof. Myeong Heon Shin, Prof. Sung-Jong Hong, Prof. Ki Sup Chung, and Prof. Chae Yoon Chon for their friendly advice.

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Written by Ji-Sook Lee

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ABBREVIATION

A : absorbance

APCs : antigen presenting cells

BCIP : 5-bromo-4-chloro -3-indolyl phosphate

BLAST : The Basic Local Alignment Search Tool

BSA : bovine serum albumin

cDNA : complementary DNA

CMV : cytomegalovirus

CTL : cytotoxic T lymphocyte

CP : cysteine protienase

DMEM : Dulbecco’s modified Eagle’s medium

DNA : deoxyribonucleic acid

EDTA : ethylenediaminetetraacetic acid

ELISA : enzyme-linked immunosorbent assay

EPG : egg per gram of feces

EST : expressed sequence tags

FABP : fatty acid binding protein

FBS : fetal bovine serum

HEK : human embryonic kidney

i.d. : intradermal

IL : interleukin

IFN : interferon

Ig : immunoglobulin

i.m. : intramuscle

NBT : nitroblue tetrazolium

NCBI : national center for biotechnology information

MHC : major histocompatibility complex

RBC : red blood cell

RNA : ribonucleic acid

RT-PCR : reverse transcriptase polymerase chain reaction

OD : optimal density

PBS : phosphate-buffered saline

PMSF : phenylmethylsulfonylfluoride

SD : standard deviation

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis

ABSTRACT

Gene expression in *Clonorchis sinensis* and development of DNA vaccine against clonorchiasis

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Clonorchis sinensis is a biliary tract parasite, which infects over 30 million people in China, Korea and Southeast Asia through the ingestion of undercooked freshwater fish that harbor the infective metacercariae. The genes expressed in *C. sinensis* adults were identified in order to develop novel drugs, better diagnostics and vaccines for the parasite. The *C. sinensis* cDNA library was constructed and DNA sequencing was performed with 450 randomly selected clones. Four hundred fifteen clones contained the amino-acid-encoding sequences. The functions of these genes could be assigned by the DNA sequence homology. The basic local alignment search tool (BLAST) analysis showed that 277 of the 415 clones were strongly matched to previously identified proteins, while the remaining 138 fell into the "no database match" category. Among the clones matching previously

identified proteins, 220 putatively identified genes were sorted into 7 functional categories. The dataset included the genes encoding the proteases, a lipid binding protein, the antigen proteins and the other genes of interest from a diagnostics, drug or vaccine development viewpoint. The present express sequence tags (EST) analysis proved to be an effective tool for examining gene expression and identified several important genes for increasing and complementing our knowledge of the biology of *C. sinensis.*

The fatty acid binding protein (FABP) has been known to play an important role in the intracellular transport of long chain fatty acids that are obtained by the fluke from the host. Although FABP has stimulated considerable interest as a vaccine target candidate, the nature of FABP from *C. sinensis* (CsFABP) remains unclear. Cysteine proteinases of *C. sinensis* (CsCP) are important virulent factors that induce pathological changes associated with larval migration, and localized biliary epithelial destruction.

This study was investigated the immunogenicity and protective efficacy of a DNA vaccine encoding CsFABP or CsCP. The CsFABP or CsCP cDNA was cloned into the expression vector pET28a, expressed in *E. coli* and the recombinant protein was purified by affinity chromatography. The recombinant CsFABP or CsCP was antigenicity with sera obtained from patients and rats with clonorchisasis. These results suggested that CsFABP or CsCP may be useful as a vaccine candidate for clonorchiasis.

Intradermal injection of plasmid DNA carrying the Cs FABP gene (pcDNA-FABP) or the CsCP gene (pcDNA3.1-CsCP) into SD rats elicited both humoral and cellular immune responses. Animals injected with pcDNA-FABP or pcDNA3.1-CsCP developed plasmid-specific antibodies which exhibited a dominance of IgG2a in sera. In addition, the DNA vaccine elicited the production of IFN-γ, but not IL-4 in splenocytes, suggesting the induction of a typical Th-1 dominated immune response in rats. The vaccination with CsFABP or CsCP resulted in a significant level of protection in SD rats against challenge infection with *C. sinensis* metacercariae. These results indicate that pcDNA3.1-CP or pcDNA3.1-CsCP induces cellular and humoral immune response. CsFABP or CsCP may be a good candidate for use in future studies of vaccination against clonorchiasis.

Keywords: *Clonorchis sinensis,* ESTs, DNA vaccine, fatty acid binding protein, cysteine proteinase, IgG2a, IFN-γ, Th-1.

CHAPTER I

Gene expression in *Clonorchis sinensis* adults using the expressed sequence tags analysis

I. INTRODUCTION

Clonorchis sinensis is an important human parasite in eastern Asia, including China, Taiwan, northern Vietnam and Korea. In Korea, it is well known that a human infection of *C. sinensis* is widely distributed along the rivers and streams.[1,2] When the fluke infects humans, the bile duct is severely dilated and the ductal wall is thickened due to mucosal hyperplasia and fibrosis. As the infection becomes chronic and heavier in intensity, complications such as obstructive jaundice, dull epigastric pain, biliary stones, ascites and cholangiocarcinoma can develop.[3,4]

Over the past decade, the world has witnessed the emergence and progress of several genome projects. The last release of the dbEST in 1999 contained a prodigious number of entries for parasite genomes including more than 20,000 ESTs for *Brugia malayi*, 12,500 for *Schistosoma mansoni*, and over 12,000 for *Trypanosoma cruzi*. In addition, an increasing number of ESTs for other parasites including *Paragonimus westermani*, *Strongyloides stercoralis*, *Toxoplasma gondii* and *Aedes aegypti* etc, have been identified. [5] The ESTs have proven to be a rapid and efficient method for characterizing the subset of genes, which are expressed in a life-stage specific manner in a wide variety of tissues and organisms.[6] Gaining knowledge on the genome of parasites has been increasingly important for understanding a parasite's

biology, the drug resistance mechanism and antigenic variations that determine the escape from a host's immune system.[7,8] Although *C. sinensis* infection is diagnosed by identifying its eggs through a microscopic stool examination, immunologic methods have recently been adopted for diagnosing *C. sinensis* infection.[9] The assembly of EST data specific to a protein or a DNA sequence can rapidly produce a gene / protein model for understanding the physical and biological properties of *C. sinensis*, and is useful for immunodiagnosis, novel drug targets and identifying potential vaccine candidates.

II. MATERIALS AND METHODS

1. Collection of parasite

The *Clonorchis sinensis* metacercariae were collected from naturally infected fish (*Pseudorasbora parva*) caught in the Nakdong River, Korea. These metacercariae were orally administered to the experimental rabbits. Eight weeks later, adult *C. sinensis* worms were obtained from the rabbit bile ducts.

2. Construction of cDNA library

C. sinensis mRNA was purified by using messenger RNA isolation kit (Stratagene, La Jolla, CA, USA). Briefly, live *C. sinensis* worms recovered from the infected rabbits were homogenized in a denaturing solution of 4 M guanidium isothiocyanate and 0.14 M β-mercaptoethanol, and centrifuged at 12,000 *g* for 10 min. The supernatant was transferred to a tube containing oligo (dT) cellulose resin. The resin was washed with a high-salt buffer, and the poly (A) +mRNA was eluted with a low-salt buffer. A cDNA library of *C. sinensis* was constructed using a ZAP Express^{TM} cDNA Gigapack Ⅱ gold cloning kit (Stratagene) according to the manufacturer's instructions. Briefly, the first strand cDNA was synthesized on 5 μg of *C. sinensis* mRNA and the second strand was synthesized by a nick translation. The

*Eco*R Ⅰ adaptors were ligated to the blunt ends. *Xho* Ⅰ restriction enzyme digestion resulted in directional cDNA. The cDNA was then inserted into the predigested ZAP express vector arms. Packaging was carried out *in vitro* with Gigapack Ⅱ packaging extract. The library was plagued on *Escherichia coli* XL1-Blue MRF'.

3. Sequencing

The phage library was converted to the plasmid form by a mass excision according to the reported protocol (Stratagene). The obtained phagemid of the library was used to infect the *E. coli* strain XLOLR. The bacteria were grown for 45 min and then plated at a low density on a medium containing a Luria-Bertani broth including tetracycline (10 ㎎/l). The bacteria were cultured at 37℃ overnight, and individual colonies were selected randomly for plasmid DNA purification and sequencing. All the sequencing reactions contained the T3 sequencing primer, and were read into the 5' end of each DNA. The reactions were run and analyzed on capillary automated sequencing machines (ABI 377 XL90, Applied Biosystem, USA). The machines generated two computer files, a chromatogram file and a plain text file.

4. Homology comparisons

Each edited EST was translated in all six reading frames and compared with the non–redundant database at the NCBI using the BLASTX program, which compares the translated nucleotide sequences with the protein sequences. The homologies to the negative reading frames, with the exception of clones with the insert in the reverse orientation, were disregarded. Putative identification for the ESTs was assigned based on the BLAST searches and in some cases with the information contained in the MEDLINE database.

III. RESULTS

1. Sequencing of ESTs

Of the 450 sequencing reactions attempted, 415 produced readable amino acid-encoding sequences from the *C. sinensis* adult cDNA library (Table 1). The leading and tailing vectors as well as the poor-quality sequences were trimmed from each text file. The 3' vector and linker sequences were removed if the poly (A^+) tails could have been included in the sequencing results. Three classes of anomalous sequences were also excluded; sequences without the insert, sequences with the reverse inserts and the incorrect adaptor. The insert size of the clones ranged from 400 to 3,000 bp, and the mean size was 627 bp.

Table I-1. ESTs of the *C. sinensis* adult cDNA library

EST match Category	No. of cDNA clone (%)
Total	*415 (100)*
Database match [a]	*277 (67)*
Putative function / domain [b]	220 (53)
No function	57 (14)
No database match [c]	*138 (33)*

[a]. 'Database match' is significant matched to other organisms (P<10(-9)). Other organisms include helminthes (*Clonorchis sinensis, Paragonimus westermani, Fasciola hepatica, Schistosoma japonicum, Schistosoma mansoni, Caenorhabditis elegans*), mammals (*Homo sapiens, Rattus norvegicus, Mus musculus*), insects (*Drosophila melanogaster*) and others.

[b]. 'Putative function / domain' is matched to the predicted or known function or protein domains

[c]. ESTs with no significant database matches are denoted as 'No database match'.

2. Putative identification of EST sequences

BLASTX analysis showed that 277 of the 415 clones were strongly matched (P<10(-9)) to other proteins. On the basis of database searches, the 277 different ESTs were classified two groups. The remaining 138 clones fell into a 'No database match' category (Table 1). The first class comprised 53% (220 clones) of the sequences with an average read of 562 ± 95 bp, and were matched to the genes with the predicted or known functions of *C. sinensis* and other organisms. Forty-one clones of these cDNAs were matched with *C. sinensis* and are related to cysteine protease, glutathione-S transferase 28 kDa and the antigen proteins. The other organisms included helminthes (*Paragonimus westermani, Fasciola hepatica, Schistosoma japonicum, Schistosoma mansoni, Caenorhabditis elegans*), mammals (*Homo sapiens, Rattus norvegicus, Mus musculus*), insects (*Drosophila melanogaster*) and others. Secondly, 14% (57 clones) of the sequences with an average sequence read of 548 ± 93 bp were matched with *Caenorhabditis elegans, Homo sapiens, Drosophila melanogaster, Mus musculus, Paragonimus westermani* with no assigned function. The remaining 33% of the sequence read of 417 ± 10 bp had no significant match in the database. The first class was sorted into 7 functional categories, which included the genes associated with energy metabolism (38), gene expression / RNA metabolism (21), regulatory / signaling components (14), protein

metabolism/sorting (98), structure / cytoskeleton (29), membrane transporter (10), and antigen protein (10) (Table 2). These were matched to either the ribosomal protein, cysteine protease or the heat shock protein. In addition, the genes related to energy metabolism were matched to those for the enzymes taking part in the glycolytic pathway, TCA cycle and oxidative phosphorylation. Among the other functional categories identified, genes encoding the proteins involved with transcription and translation and those associated with the regulatory and signaling function were identified.

Table I-2. Significant matches of *C. sinensis* adult ESTs with sequences present in DNA and protein databases.[a]

EST	Accession No.	Putative identification	Species	No. of clones	Length EST[b](bp)	e-value
Structure/Cytoskeleton (29)						
CS478	AT006944	actin	*Crassostrea gigas*	1	624	e-105
CS128	AT006733	alpha tubulin	*Schistosoma mansoni*	5	653	e-109
CS8	AT006749	alpha tubulin chain	*Lytechinus pictus*	2	564	2e-47
CS467	AT006945	alpha-1 tubulin	*Hirudo medicinalis*	1	654	3e-88
CS72	AT006728	alpha-3 tubulin chain	*Drosophila melanogaster*	1	668	e-110
CS28	AT006762	alpha-tubulin	*Xenopus laevis*	1	630	3e-80
CS98	AT006730	beta-1 tubulin chain	*Physarum polycephalum*	1	635	3e-83
CS214	AT006740	beta-tubulin	*Halocynthia roretzi*	5	549	7e-83
CS200	AT006851	dynein light chain	*Schistosoma mansoni*	1	395	3e-21
CS232	AT006864	fibrillar collagen	*Arenicola marina*	1	603	7e-15
CS334	AT006946	fragile X-related protein	*Danio rerio*	1	632	2e-06
CS38	AT006947	inner dynein arm right chain	*Strongylocentrotus purpuratus*	1	439	1e-39

CS469	AT006948	intermediate chain 1	*Anthocidaris crassispina*	1	623	4e-33
CS220	AT006859	paramyosin related protein	*Echinococcus granulosus*	1	553	3e-07
CS185	AT006840	prosaposin precursor	*Danio rerio*	1	607	6e-06
CS331	AT006949	testicular microtubules-related protein	*Homosapiens*	1	393	1e-22
CS433	AT006950	thrombospondin precursor	chicken	1	602	2e-13
CS475	AT006951	vitelline protein B1	*Fasciola hepatica*	1	560	7e-30
CS177	AT006834	vitelline protein B2	*Fasciola hepatica*	1	608	1e-21
CS471	AT006937	female genital complex protein	*Fasciola hepatica*	1	547	1e-13
Membrane transporter (10)						
CS328	AT006912	hexosaminidase B	*Mus musculus*	1	626	2e-28
CS118	AT006800	integral membrane protein 23	*Schistosoma mansoni*	1	653	2e-22
CS290	AT006894	Na+/K+-exchanging ATPase beta chain	Sheep	1	658	1e-26
CS367	AT006952	nucleoporin 153	*Takifugu rubripes*	1	524	1e-07
CS154	AT006818	oxalate/formate antiporter	*Archaeoglobusfulgidus*	1	572	3e-06
CS248	AT006870	Propionyl Coenzyme A carboxylase	*Mus musculus*	1	643	3e-49

CS159	AT006820	Sodium-dependent amino acid transporter	*Chlamydia trachomatis*	1	626	2e-15
CS401	AT006953	transmembrane protein	*Caenorhabditis elegans*	1	646	7e-20
CS22	AF527455	V-ATPase G subunit	*Caenorhabditis elegans*	1	560	1e-09
CS89	AT006786	ER lumen protein retaining receptor	*Drosophila melanogaster*	1	733	4e-91
Gene expression/ RNA metabolism (21)						
CS181	AT006838	14-3-3 protein epsilon	*Xenopus laevis*	1	605	3e-38
CS141	AT006811	asparaginyl-tRNA synthetase	*Homo sapiens*	1	659	5e-47
CS436	AT006925	aspartyl-tRNA synthetase	*Rattus norvegicus*	1	489	2e-27
CS438	AT006926	casein kinase II alpha subunit	*Spodoptera frugiperda*	1	543	1e-81
CS209	AT006738	DNA supercoiling factor	Silkworm	1	659	3e-24
CS202	AT006853	DnaJ-like protein 1	*Mus musculus*	1	655	1e-04
CS244	AT006743	GTP-binding protein	*Discopyge ommata*	1	650	2e-73
CS473	AT006927	human translation initiation factor 6	*Homo sapiens*	1	554	4e-66
CS400	AT006928	Kruppel homolog 2	*Drosophila melanogaster*	1	462	4e-06
CS91	AT006729	microrchidia	*Mus musculus*	1	636	3e-42

CS497	AT006929	mitochondrial dicarboxylate carrier	*Mus musculus*	1	559	2e-31
CS278	AT006888	phenylalanine-tRNA synthetase-like protein	*Homo sapiens*	1	663	5e-32
CS115	AT006798	SET translocation	*Homo sapiens*	1	659	6e-69
CS458	AT006931	small glutamine-rich tetratricopeptide	*Homo sapiens*	1	556	2e-29
CS207	AT006855	small nuclear ribonucleoprotein D1 polypeptide	*Homo sapiens*	1	548	2e-33
CS5	AT006746	small zinc finger-like protein	*Rat*	1	316	3e-07
CS59	AT006777	splicing factor 3b, subunit 2, 145kD	*Homo sapiens*	1	632	1e-05
CS24	AT006760	TB2	*Homo sapiens*	1	586	6e-39
CS461	AT006932	transcription regulatory protein	*Oryza sativa*	1	617	1e-12
CS543	AT006933	Y-box binding protein homolog	*Schistosoma mansoni*	2	517	1e-31
Signaling Components (14)						
CS63	AF480464	adenylate kinase	*Schistosoma mansoni*	1	648	5e-73
CS73	AT006780	Ca2+/Calmodulin-dependent protein kinase I	*Homo sapiens*	1	646	8e-37

CS296	AF527456	calcium-binding protein	*Fasciola hepatica*	1	362	8e-15
CS297	AT006954	caltractin	*Homo sapiens*	1	651	2e-15
CS349	AT006955	cAMP-dependent protein kinase	*Caenorhabditis elegans*	1	619	8e-70
CS341	AT006956	Chlamydomonas radial spoke protein 3	*Homo sapiens*	1	634	2e-29
CS313	AT006907	dopamine responsive protein	*Rattus norvegicus*	1	560	2e-47
CS38	AT006957	GTP:AMP phosphotransferase	*Bos taurus*	1	468	4e-26
CS191	AT006844	GTP-binding protein ara2	*Arabidopsis thaliana*	1	602	4e-67
CS114	AT006797	Pka-C1 gene product	*Drosophila melanogaster*	1	653	8e-71
CS413	AT006958	programmed cell death 6	*Homo sapiens*	1	628	2e-36
CS176	AT006833	senescence-associated protein	*Pisum sativum*	1	628	4e-42
CS323	AT006911	SH3-domain kinase binding protein 1	*Homo sapiens*	1	588	1e-20
CS298	AT006898	vesicle -associated membrane protein 2	*Xenopus laevis*	1	642	2e-16
Protein metabolism/sorting (98)						
CS129	AT006804	brain specific protein	*Homo sapiens*	1	630	2e-16
CS268	AT006884	calpain	*Drosophila melanogaster*	1	647	6e-72
CS4	AF093242	cysteine protease	*Clonorchis sinensis*	30	678	e-119

CS227	AT006862	cysteine and histidine-rich protein	*Mus musculus*	1	603	1e-05
CS96	AT006934	cysteine-rich protease inhibitor	*Mus musculus*	1	619	3e-14
CS47	AT006772	cytosol aminopeptidase	*Schistosoma mansoni*	1	501	8e-15
CS554	AT006936	elongation factor Tu homologue	*Caenorhabditis elegans*	1	590	7e-47
CS370	AT006937	elongation factor-1a-related protein	*Anthocidaris crassispina*	2	434	1e-70
CS308	AT006904	eukaryotic translation initiation factor 4	*Homosapiens*	1	662	1e-06
CS33	AT006765	ferritin-2 heavy chain	*Schistosoma mansoni*	1	496	7e-45
CS261	AT006879	glycoprotein 96-92	*Leishmania major*	1	645	6e-08
CS282	AT006891	granulin	*Homo sapiens*	1	448	9e-11
CS119	AT006801	heat shock protein 110	*Strongylocentrotus franciscanus*	1	657	5e-55
CS16	AT006755	heat shock protein 60	*Drosophila melanogaster*	2	604	4e-77
CS496	AT006938	heat shock protein 67B2	*Mus musculus*	1	489	3e-12
CS546	AT006939	heat-responsive protein	*Mus musculus*	1	498	9e-38
CS521	AT006940	heme binding protein 2	*Mus musculus*	1	553	9e-22
CS465	AT006941	hemoglobinase	*Schistosoma japonicum*	1	544	4e-42
CS510	AT006942	Hsp89-alpha-delta-N	*Homo sapiens*	2	563	5e-94
CS474	AT006943	mago-nashi(Drosophila) homolog	*Homo sapiens*	1	346	9e-36

CS459	AT006959	metaxin 2 (mitocondial memb.)	*Mus musculus*	1	583	6e-04
CS26	AT006761	mitochondrial ribosomal protein L2	*Drosophila melanogaster*	1	624	3e-21
CS507	AT006960	preprocathepsin C	*Schistosoma japonicum*	1	610	4e-34
CS56	AT006775	ribosomal protein L11 60S	*Caenorhabditis elegans*	1	596	2e-72
CS195	AT006847	prolyl endopeptidase	*Sus scrofa*	1	604	7e-55
CS133	AT006734	polyubiquitin	*Suberites domuncula*	2	528	3e-72
CS212	AT006739	ribosomal protein L3 60S	*Toxocara canis*	1	598	1e-69
CS466	AT006961	ribosomal protein L34 60S	*Aedes albopictus*	1	443	2e-37
CS315	AT006909	ribosomal protein L36 60S	*Homo sapiens*	1	315	3e-20
CS442	AT006962	ribosomal protein S1	*Xenopus laevis*	1	523	4e-69
CS479	AT006963	ribosomal protein S10 40S	*Ictalurus punctatus*	1	551	1e-24
CS36	AT006768	ribosomal protein S15-B 40S	*Schizosaccharomyces pombe*	1	466	1e-36
CS105	AF527457	ribosomal protein S24	*Mus musculus*	2	469	2e-33
CS483	AT006964	ribosomal protein S4	*Gallus gallus*	1	593	3e-78
CS18	AT006757	ribosomal protein S4 40S	Chicken	1	602	2e-85
CS184	AT006735	ribosomal protein S8 40S	*Homo sapiens*	2	603	2e-58
CS206	AT006854	sec23 gene product [alt 1]	*Drosophila melanogaster*	1	648	2e-80
CS62	AT006778	Sec24D protein like protein	*Drosophila melanogaster*	1	641	3e-36

CS455	AT006965	senescence-associated protein	*Pisum sativum*	1	561	1e-46
CS443	AT006966	thrombospondin	*Gallus gallus*	2	602	2e-13
CS374	AT006967	transaldolase 1	*Homo sapiens*	1	439	2e-44
CS80	AT006785	translation initiation factor 2, subunit 2 (beta)	*Homo sapiens*	1	642	1e-12
CS31	AT006764	Transpanin like protein	*Caenorhabditis elegans*	1	602	2e-08
CS517	AT006968	ubiquitin	*Caenorhabditis elegans*	1	513	5e-74
CS404	AT006969	ubiquitin-conjugating enzyme E2 variant 2	*Homo sapiens*	1	573	3e-43
CS391	AT006970	acute morphine dependence related protein 2	*Homo sapiens*	1	458	1e-54
CS450	AF051318	glutathione-S transferase 28kDa	*Clonorchis sinensis*	7	574	1e-37
CS430	AT006971	mitochondrial thioredoxin	*Bos taurus*	1	550	3e-30
CS360	AT006972	proteasome (prosome, macropain)	*Homo sapiens*	1	540	6e-44
CS369	AT006973	ubiquitin-conjugating enzyme	*Homo sapiens*	1	433	1e-59
CS78	AT006784	myoglobin	*Paramphistomum Epiclitum*	5	568	4e-28
CS243	AT006869	B7	*Homo sapiens*	1	649	8e-26

Metabolism (38)

CS501	AT006914	4-Hydroxybutyrate CoA-transferase	*Clostridium aminobutyricum*	1	594	3e-40
CS482	AT006915	aldehyde dehydrogenase (NAD+)	*Enchytraeus buchholzi*	1	572	1e-52
CS250	AT006872	alpha-mannosidase C1orf22	*Homo sapiens*	1	645	2e-07
CS50	AT006774	alpha-propionyl-CoA carboxylase	*Rattus norvegicus*	1	506	2e-27
CS139	AT006809	carbonic anhydrase II	Bovine	2	656	1e-39
CS519	AT006916	Citrate Synthase Complex	*pig*	1	515	8e-64
CS163	AT006823	coproporphyrinogen III oxidase	*Drosophila melanogaster*	1	628	7e-82
CS539	AT006917	Cu/Zn-superoxide dismutase	*Fasciola hepatica*	1	551	8e-59
CS417	AT006918	cytochrome b5	*Oryctolagus cuniculus*	1	582	2e-26
CS307	AT006903	cytochrome c	*desert locust*	1	565	8e-47
CS368	AT006974	cytochrome c oxidase subunit 2	*Fasciola hepatica*	2	527	1e-28
CS312	AT006906	cytochrome c oxidase subunit I	*Oryctolagus cuniculus*	2	533	2e-42
CS194	AT006846	dihydrolipoamide S-acetyltransferase	*Homo sapiens*	1	605	3e-28
CS306	AT006902	NAD+ oxidoreductase	*Canis familiaris*	1	635	3e-50
CS213	AF480465	enolase	*Ricinus communis*	2	656	1e-68
CS92	AF527454	fatty acid-binding protein	*Schistosoma japonicum*	1	578	1e-30

CS77	AT006783	formiminoglutamate hydrolase	*Streptococcus pyogenes*	1	647	7e-09
CS500	AT006919	fructose-1,6-bisphosphate aldolase	*Echinococcus multilocularis*	1	454	1e-41
CS167	AT006737	Glyceraldehydes-3-phosphate dehydrogenase	*Chondrus crispus*	1	625	6e-79
CS131	AT006806	glyceraldehyde-3-phosphate dehydrogenase	*Schistosoma japonicum*	1	644	2e-23
CS415	AT006920	glycerol kinase	*Mesorhizobium loti*	1	618	5e-14
CS74	AT006781	liver glycogen phosphorylase	*Rattus norvegicus*	1	660	3e-50
CS252	AT006873	malate dehydrogenase	*Drosophila melanogaster*	2	646	7e-72
CS376	AT006921	NADH dehydrogenase subunit 4	*Paragonimus westermani*	1	240	6e-21
CS15	AT006754	NADH dehydrogenase subunit 6	*Paragonimus westermani*	1	100	1e-08
CS271	AT006731	phosphoglycerate mutase	*Schistosoma japonicum*	1	567	3e-80
CS49	AT006773	phosphoglycerate mutase (gpmA)	*Lyme disease spirochete*	1	453	2e-32
CS502	AT006922	dehydrogenase kinase 4	*Mus musculus*	1	654	5e-29
CS14	AT006753	Rieske Fe-S protein precursor	*Rattus norvegicus*	1	643	1e-18
CS527	AT006923	succinyl-CoA synthetase beta-subunit	*Sus scrofa*	1	537	7e-35
CS101	AT006790	tyrosinase	*Pelodiscus sinensis japonicus*	1	640	3e-21

CS339	AT006924	uridine phosphorylase	*Homo sapiens*	2	627	5e-44
Antigen protein/ Others (10)						
CS276	AF136608	antigen Cs44	*Clonorchis sinensis*	3	515	4e-27
CS42	AT006769	autoantigen	*Rhipicephalus appendiculatus*	1	462	3e-07
CS153	AT006817	Ly6-C antigen	*Rattus norvegicus*	2	594	2e-06
CS239	AF343876	proline-rich antigen	*Clonorchis sinensis*	1	444	9e-34
CS231	AT006742	Sj-Ts4 protein	*Schistosoma japonicum*	1	580	1e-18
CS39	AT006727	sperm associated antigen 6	*Mus musculus*	1	467	2e-54
CS9	AT006744	tegumental antigen Sm20.8	*Schistosoma mansoni*	1	681	2e-07

[a]. Matches were sort into functional categories and only one representative match is given. Length ESTs are in nucleotides of reading sequences.

IV. DISCUSSION

Even though the identification of novel parasite molecules using conventional methods is tedious, identifying some of these molecules might serve as starting points for the various studies. However, the searches for *C. sinensis* gene expression using the EST analysis approach performed in this study, showed many important genes for increasing and complementing our knowledge of the biology of *C. sinensis.*

Energy production in this fluke depends largely on glycolysis.[9,10] Through glycolysis, it takes up approximately 1.13 mg of glucose/hour/g of wet weight, produces lactate, and forms several types of amino acids as the end product from exogenous glucose.[10,11] Therefore, the enzymes involved in glycolysis are essential for energy metabolism. In this work, the ESTs found exhibited a variety of proteins related to metabolism. They were suggested to produce ATPs through the glycolytic pathway or aerobic metabolism (Table 2; AT006919, AT006737, AT006806, AT006873, AF480465, AT006919, 006731, AT006921). However, an initial search of the genomic basis of the fundamental biochemical pathways of *C. sinensis* revealed that its biosynthetic networks are fairly consistent with those of humans.

The adenylate kinase 1 (AK 1, AF480464) homologous gene was identified from the ESTs. AK 1 is indispensable for *Escherichia coli*[12] and *Schizosaccharomyces pombe* growth[13], indicating that it is an essential

enzyme for life in a single cell. Under normal conditions, the AK 1- knockout mice showed no phenotypical changes. However, under metabolic stress, compromised energetics was detected in the heart and skeletal muscle, suggesting the physiological significance of AK-catalysed phosphoryl transfer between the intracellular compartments in cellular energetic homoeostasis (Qualtieri et al. 1997; Janssen et al. 2001).[14, 15] The nucleoside analogs of AK have been used clinically for treating certain viral infections and malignant diseases.[16,17,18] Therefore, it can be also targeted for drug research with recombinant antigens.

Several genes encoding antioxidant and detoxification enzymes, such as Cu/Zn-superoxide dismutase, and glutathione-S-transferase were identified. These proteins are believed to play a crucial role in protecting the parasite from the host immune effector mechanisms, and are being pursued as drug targets in other parasitic infections.[19,20] Of protease genes identified from EST analysis, the high frequency of cysteine protease gene expression (30 out of 415 randomly selected clones) suggests an important role of cysteine protease in the metabolism and/or pathogenesis of clonorchiasis. This has also been proposed as a target for a structure-based approach for drug design.[21,22] On the other hand, one protease inhibitor was also identified. Specific protease inhibitors have also been isolated from filarial nematodes, and are suggested to play a role in inhibiting the enzymes secreted from host immune cells, the

blocking of antigen processing and the control of the endogenous proteases involved in parasite development.[23,24] The biological role of the protease inhibitor in *C. sinensis* requires further investigation. The ESTs identified in this study showed a significant number of homologous genes previously reported from closely related parasitic trematodes. A homologue of the *S. japonicum* fatty acid binding protein (FABP) was identified in *C. sinensis*, and it was demonstrated that the FABP with a cross-protective efficacy could be used as a vaccine against trematode infections, such as fasioliasis[25-27] and schistomiasis.[28] It was also found that the ESTs contained several antigenic protein genes for *C. sinensis* or other parasites. The recombinant glycine rich *C. sinensis* protein or proline-rich antigen was reported previously to be useful for immunodiagnosis with a high specificity.[29,30]

AT006742 and AT006744 were found to be homologous to the *Schistosoma* tegumental antigens. Sm 20.8 was reported to be a member of a family of soluble tegument antigens that contained the EF-hand motifs, and were recognized as antigenic targets with protective antisera.[31] The most significant feature of the flatworm tegument is that the ultimate boundary between the parasite and host is a living plasma membrane and its associated polyanionic coating or glycocalyx. This fact has revolutionized the understanding of symbiotic relationships, in that it raises a new conceptual level of the significance of the host-parasite relationship. *In vivo*, the

alimentary tracts in schistosomes and the liver fluke play a role primarily in macromolecular digestion and the subsequent absorption of soluble digestive products. However, it is likely that this is augmented by the host-derived sugars and amino acids absorbed by the tegument.[32] This can be also a target for developing diagnostics with recombinant antigens.

The EST analysis using the various stages of the parasite life cycle, such as metacercariae, which is the infective stage to the final host, will be useful for investigating gene expression and characterizing the purified proteins of interest in *C. sinensis.* A particular gene or a class of genes identified within the EST dataset can be used an appropriate target for a diagnostics, vaccines or drug research and the development of clonorchiasis in the future.

CHAPTER II

DNA vaccination confers protective immunity to rats infected with *C. sinensis*

I. INTRODUCTION

Protein-based vaccines are usually co-administered with immunologic adjuvants which deposit the antigen and stimulate a non-specific inflammatory response. The most effective adjuvants are often unsuitable for human use because of their toxicity. Another approach to vaccination is based on direct intramuscular (i.m.) or intradermal (i.d.) injection of a DNA expression plasmid encoding the antigen.[33,34] Early observations demonstrated that a gene under the control of a proper promoter can elicit an immune response in mammals. The DNA-based vaccine technology is a promising new tool in the development of vaccines and has been shown to confer immunity against different infectious diseases including parasitic infections.[35-38] The mechanisms of action of DNA vaccines have been mainly investigated in mice using either the i.d. or i.m. routes of immunization. I.m. injection results in low-level transfection of myocytes whereas i.d. injection may direct ly transfect antigen presenting cells (APCs).[39,40] Because myocytes express major histocompatibility complex (MHC) class I at a low levels and do not constitutively express MHC class II or costimulatory molecules such as B7. Immune responses are resulted by priming with different cell types, immunization route and methods.[41] A variety of important genes expressed *C. sinensis* adult worm were identified by EST analysis in chapter I. I have now tested two further antigen, fatty acid binding protein and cysteine proteinase

from *C.sinensis*, for their potential to elicit cellular and humoral response when delivered as DNA vaccines.

The fatty acid binding proteins (FABPs) are a multigenic family of cytoplasmic proteins of low molecular mass (14-15kDa). They have specific tissue distribution and are differentially regulated during development.[42] It has been demonstrated that the *Fasciola* FABP was effectiveness in vaccines against trematode infections, including *F. hepatica*[25], *Schistosoma mansoni*[26], and *F. gigantica*.[27] Furthermore, an FABP homologue from *S. mansoni* is able to protect experimental animals infected with both *S. mansoni* and *F. hepatica*.[28] These findings suggested that this molecule could form the basis of a single vaccine against multiple parasitic infections.

Another promising set of vaccine antigens against clonorchiasis is the cystiene proteinase. The majority of unicellular and multicellular parasites that infect mammalian hosts produce a wide variety of proteinases, some of which are secreted. The functions of these proteinases are varied and proposed to be for the purposes of feeding tissue migration, and perhaps immune envasion.[43] It has been suggested that the cysteine proteinase in the adult worms of *Schistosoma mansoni* [44], *Fasciola hepatica*[45], and *Clonorchis sinensis*[46] hydrolyze hemoglobin and collagen and may play an important role in the acquisition and metabolism of nutrients. Cysteine proteinases showed strong antigenicty against sera from patient with clonorchiasis.[46-49]

This proteinase as a vaccine candidate and a source of protective antigens, was one of the major molecule of *Fasciola hepatica*, *Leishmania major*, *Schistosoma japonicum*.[37,50-54] The intramuscular immunization of rats with the *F. hepatica* CP cDNA protected against infection by metacercariae of *F. hepatica* the fluke. [37]

C. sinensis induces clear systemic and local immune responses in infected hosts although they differ considerably in respect of capability to develop protection following subsequent infection. The major problem linked with inducing protective immune response against *C. sinensis* is that the precise mechanisms of the protection against this parasite have not been elucidated. Although a drug such as praziqantel is effective in disease caused by this fluke[55], the cost of treatment with this drug prohibits its wide adoption by rural people in developing countries. There is need to develop alternative, cost effective, and sustainable strategies, such as a vaccine, for control of clonorchiasis.

The cDNA encoding FABP of *C. sinensis* and cDNA encoding CP of *C. sinensis* have been found in EST analysis, which is considered as a vaccine candidate in clonorchiasis and other parasitic infections. In this study, we describe the immunogenicity and protective capacity of plasmid DNA immunization encoding CsFABP and CsCP.

II. MATERIALS AND METHODS

1. Sequence analysis and alignment of CsFABP and CsCP

The cDNA encoding FABP and CP were obtained by EST analysis of adult worm *C. sinensis* cDNA library which was constructed using a ZAP Express™ cDNA Gigapack Ⅱ gold cloning kit (Stratagene, La Jolla, CA). Complete protein sequences were aligned with the Clustal X program (ftp;//ftp-igbmc.u-strasbg.fr/pub/ClustalX).

2. Expression and purification of recombinant CsFABP and CsCP

FABP : CsFABP cDNA containing the coding region and 3'-untranslated region was double digested with *Bam* HI / *Xho* I endonucleases and subcloned into the corresponding restriction sites of prokaryotic expression vector, pET 28a (Novagen, Madison, WI). CsFABP-pET28a was expressed in *E.coli* BL21 (DE3) (Promega, Madison, WI). The transformed bacteria were grown in LB medium at 37°C. Growth was monitored by absorbance at 600 nm. The cells were induced with 1.0 mM IPTG at OD600 = 0.6 expression and grown overnight at 25°C. The harvested cells (wet weight; 4 g/liter) were either stored at -70°C or lysed on ice in 30 ml of Buffer A (50mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole) containing 0.5 mg/ml lysozyme. After 30 min of incubation, the cells were sonicated and

then centrifuged at 15,000 *g* for 1 hr at 4°C. The supernatant was collected and incubated with Ni^{2+}-NTA agarose (Qiagen, Valencia, CA) resin (2.5 ml of resin for 1 liter of induced cells) equilibrated in Buffer A and shaked gently for 1 hr to allow binding. The supernatant / agarose mixture was poured into a column and the bound protein and resin were washed with 10 column volume of Buffer A, as well as 10 column vol of increasing concentrations of 20 mM imidazole and 3 column volume of 250 mM imidazole. Protein purity was monitored by Coomassie blue staining after SDS-PAGE and the purified protein was concentrated using Centriprep YM-10 (Amicon, Beverly, MA). Protein concentration was estimated using the Bradford assay (Bradford, 1976). Aliquots of the purified recombinant CsFABP (rCsFABP) were stored at -70° until used for preparation of anti-rFABP antibody, enzyme linked-immunosorbent assay (ELISA) or for *in vitro* stimulation of splenocytes.

CP : CsCP cDNA containing the coding region and 3'-untranslated region was double digested with *Eco* RI / *Xho* I endonucleases and subcloned into the corresponding restriction sites of prokaryotic expression vector, pET 28a (Novagen, Madison, WI). CsCP-pET28a was expressed in *E.coli* BL21 (DE3) (Promega, Madison, WI). The transformed bacteria in each case were grown in LB medium at 37°C. Growth was monitored by absorbance measurements at 600 nm (OD600) and at OD600=0.6 expression was

induced with 1.0 mM IPTG. The cells were grown overnight at 25°C. Subsequently, bacteria were sedimented (6000 *g*, 30min) and the pellet was resuspended in 50 mM phosphate buffer, 300 mM NaCl, pH 8.0 (2 ml buffer per g of cells), supplemented lysozyme (1 mg/ml) and incubated on ice for 30 min. Cells were sonicated and then centrifuged at 15,000*g* for 1h min at 4°C. The pellet was washed with 50 mM phosphate buffer, 300 mM NaCl, 0.5 % Triton X-100, pH 8.0. Sedimented inclusion bodies were solubilized using 4 volumes of Buffer A (6 M guanidine-HCl, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0) supplemented with 10 mM imidazole. After solubilization and sedimentation of particulate materials at 6000 *g* for 10 min, the recombinant CsCP (rCsCP) was purified Ni^{2+}-NTA agarose resin following the manufacturer's recommendations for purification under denaturing conditions with minor modifications (Qiagen GmbH, Hilden, Germany). The solubilized inclusion bodies from approximately 2.5 liters of bacteria culture were subjected to 3ml of packed Ni^{2+}-NTA resin column equilibrated with buffer A. The column was subsequently washed with 100 volumes buffer A, 10 volumes buffer B (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0), 10 volumes buffer C (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 6.3) supplemented with 10 mM imidazole and 10 vol of buffer C supplemented with 20 mM imidazole. The protein was eluted with buffer C supplemented with 500 mM imidazole. Aliquots of fractions

were analyzed on 12% separation gels. Fraction containing purified rCsCP were pooled and diluted with 1 volume of buffer C. Subsequently, rCsCP was slowly diluted by dropwise addition to a 100-fold volume of refolding buffer [50 mM Tris-HCl, 300 mM NaCl, 5% glycerol, 0.1 mM EDTA, 0.5 mM oxidized glutathione (GSSG), 3 mM reduced glutathione (GSH), pH6.8]. Refolded and soluble rCsCP was concentrated using Centriprep YM-10 (Amicon, Beverly, MA) and finally determined by the Bradford method. [56] liquots of the purified rCsCP were stored at -70°C until used for enzyme-linked immunosorbent assay (ELISA) or for in vitro stimulation of splenocytes.

3. Preparation of rat anti-rCsFABP antisera and rat anti-rCsCP

The rCsFABP (1 mg/ml) and rCsCP (1 mg/ml) was respectively each mixed with an equal volume of complete Freund's adjuvant, and 400 μl of this mixture was injected into the peritoneum of female Sparue-Dawley rats (6 weeks old). After 2 weeks, the mice were injected peritoneally with an equal amount of the antigen mixed with incomplete Freund's adjuvant. Another 2 weeks later, 200 μl of the aqueous antigen was injected into a tail vein of the rats. After 3 days of the last immunization, blood was collected from the orbital sinus of the rats, and the sera isolated were pooled.

4. Antigenicity of rCsFABP and rCsCP

The purifed rCsFABP and rCsCP were respectively separated on 12 % SDS-PAGE PAGE. Afterwards, the gels were transferred to a 0.45 μm nitrocellulose membrane (Hybond ^{TM}-C, Amersham Pharmacia Biotech, Uppsala, Sweden) as previously described.[57]e membranes were proved with rat anti-rCsFABP antisera, rat anti-rCsCP antisera, sera from patients with serologically proven clonorchiasis, sera from rat with infected *C. sinensis* at 1:200 dilution. Alkaline phosphatase conjugated anti-human or anti-rat IgG antibody (Sigma, St. Louis, MO) was used as a secondary antibody at 1:2000 dilution. Color was developed with BCIP/NBT substrates (Sigma).

5. Construction of a DNA vector expressing CsFABP and CsCP

The gene for CsFABP and CsCP were respectively obtained from a pBK-CMV genomic library of *C. sinensis* and was cloned into pET28a.

FABP : A 576bp fragment containing the CsFABP gene and its promoter sequences were excised from the insert of pET28a with *Bam* HI and *Not* I restriction enzyme digestion and ligated into expression vector pcDNA3.1 downstream to the CMV promoter (Invitrogen, San Diego, CA.). The resulting plasmid was designated as a pcDNA3.1-FABP. A colony of *E. coli*

DH5α containing pcDNA3.1-FABP was cultured in Luria-Bertani broth containing ampicillin (100 μg/ml).

CP : Part of the gene coding for the pro-form of CsCP (amino acid residues 2-326) was emplified by PCR using the oligonucleotide primers CsCP-Fk (5'-ACCATGGGCGACTTTTCGTGTGC) and CsCP-R (5'-CTATTTGATAATCGCTGTAGT). The PCR was carried out with initial denaturation at 95°C for 2 min, then 30 cycles of denaturation at 94°C for 30sec, annealing at 52°C for 30 sec, and extension at 72°C for 1min. The PCR product was cloned into pGEM-T easy vector (Promega), subsequently with *Eco* RI restriction enzyme digestion and ligated into expression vector pcDNA3.1 downstream to the CMV promoter (Invitrogen, San Diego, Calif.). The resulting plasmid was designated pcDNA3.1-CsCP. A colony of *E. coli* DH5α containing pcDNA3.1-CsCP was cultured in Luria-Bertani broth containing ampicillin (100 μg/ml).

Large-scale plasmid DNA isolation was performed using an EndoFree Plasmid Maxi Kit (Qiagen, Valencia, Calif.) according to the manufacturer's directions. The DNA was finally resuspended in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml. DNA concentration and purity were determined by optical density; the A_{260}/A_{280} ratio was typically greater than 1.9. The DNA plasmid constructs were verified by restriction digestion and

by sequencing the complete insert with capillary automated sequencing machines (ABI 377 XL90, Applied Biosystem, USA).

6. Transfection of mammalian cells

Plasmid constructs were tested for expression of protein in HEK 293 cells prior to being used in rats as DNA vaccines. Freshly grown HEK 293 cells seed at 1×10^5 per 35 mm dish in 2 ml of DMEM media containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and incubated in 5% CO_2 at 37°C until 80-90% confluent. Transfection was facilitated using Lipofectamine 2000 (Life technologies, Grand Island, NY), according to the manufacturer's instructions. The total amount of 1 µg of an empty vector pcDNA3.1 or pcDNA3.1-FABP or pcDNA3.1-CP and 2 µl of Lipofectamine 2000 was used in each transfection. The cells were harvested after 48 hr of transfection, and then lysed in homogenization buffer [10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, and protease inhibitors]. Protein samples (15 µg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. The blots were incubated with rat anti-rCsFABP antibody or rat anti-rCsCP antibody and developed using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech,

Piscataway, NJ).

7. Collection of metacercariae

Pseudorasbora parva harbouring the metacercariae of *C. sinensis* were captured in Nakdong-River, Korea, an endemic area. Whole fish muscle was removed and digested in artificial gastric juice for half an hour at 37°C. The digested materials were filtered the sieve and washed several times with saline. *C. sinensis* metacercariae were then identified and collected under a stereoscopic microscope.

8. Experiment animals

Female 6-week-old Sprague-Dawley (SD) rats were used for the vaccination experiments. To confirm the *in vivo* expression of CsFABP or CsCP and protection experiments, rats were anesthetized with inhaled halothane and injected with 200 μg of plasmid DNA in 200 μl of PBS into the shaved dorsal skin of animals using a syringe with a 28-gauge needle. Rats were vaccinated two times at 2 week intervals before injection, with pcDNA3.1-FABP or pcDNA3.1-CP. As negative controls, groups of rats were immunized with pcDNA3.1 without insert or with PBS only (Table 1). All animals were individually numbered and marked. During the course of experiment rats were housed in commercial rat cages with grid floors, and feed a commercial rodent diet. All rats were bled by the tail vein before the

vaccination and thereafter at 2 week intervals. Sera were stored at -20°C until further use.

Table II-1. Experimental protocols used for the five experimental groups

Group	No.	Treatment	-5 wk	-3 wk	-1 wk	0 wk	7 wk
uninfected	6	Non	-	-	S(3)		S(3)
Infected only	10	PBS	1’	2’	S(5)	Inf.	S(5)
Vector only	10	pcDNA3.1	1’	2’	S(5)	Inf.	S(5)
Vaccinated FABP	10	pcDNA3.1-FABP	1’	2’	S(5)	Inf.	S(5)
Vaccinated CP	10	pcDNA3.1-CP	1’	2’	S(5)	Inf.	S(5)

Inf., animals in group were infected with 50 metacercariae *C. sinensis*; number in parenthesis, rat number of sacrifice

9. Challenge infections and parasite loads

Immunized rats received a challenge infection 3 weeks after the final DNA injection. Fifty *C. sinensis* metacercariae were administrated orally through a gavage needle to anaesthetized rats. At 15 days after challenge infection, stool egg examination was performed by formalin-ether sedimentation.[58] The number of eggs per gram of feces (EPG) was counted[59] every three or five days until the 49th day (7 weeks) of infection. At seven weeks after the infection, rats were sacrificed, and adult *C. sinensis* were recovered from the bile duct.

10. ELISA

The presence of serum immunoglobulin G (IgG), IgG1, IgG2a, and IgE isotypes specific to CsFABP or CsCP was determined by indirect ELISA. The rCsFABP or CsCP was diluted to 5 μg/ml in carbonate buffer (pH 9.6) and used to coat the wells of a polystyrene modular strip (Costar, Corning, USA). After overnight incubation at 4°C, plates were washed four times with PBST (Phosphate-buffered saline [pH 7.0] with 0.05% Tween 20). For blocking non-specific binding, plates were inoculated with 3% BSA (bovine serum albumin, Sigma) in PBS for 1 hr at 37°C and then incubated with serial dilution of the sera for 2 hr at room temperature and washed four times. Isotype-specific anti-rat horseradish peroxidase conjugates (Bethyl Lab.Inc,

Montgomery, TX.) were added (100 μl/well) at an appropriate dilution. After 1 hr incubation at room temperature, plates were washed four times, and 100 μl of substrate solution (3,3',5,5'- tetramethyl-benzidine; TMB, 0.4 g/L; KPL, Gaitherburg, ML.) was added to each well. After 20 min incubation at room temperature, the enzyme reaction was stopped by addition of 100 μl of stop solution, and the absorbance of the developed color was measured at 450 nm with an automatic microplate reader (TECAN, Salzburg, Austria). The cut-off value for the assay was calculated as the mean specific optical density plus standard deviation (SD) for 5 sera from non-immunized rat assayed at a dilution of 1: 100. The titer of each serum was calculated as the reciprocal of the highest serum dilution yielding a specific optical density higher than the cutoff value.

11. Splenocyte preparation

Rats were sacrificed before infection and at 7 weeks after infection, and their spleen were removed under aseptic conditions. Single-cell suspensions were prepared from the spleens according to the standard procedure[60], and the red blood cells were removed using RBC lysis solution (Sigma). Splenocytes were cultured in 96-well flat-bottom plate at a concentration of 5×10^6 cells/well in the presence of 5 μg of crude extract *C. sinensis* adult worms, 1 μg of purified rCsFABP, or rCsCP, or PBS (unstimulated control).

RPMI 1640 medium (Sigma) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (GibcoBRL, Rockvile, MD), and 100 U/ml of penicillin-streptomycin was used for culturing the splenocytes.

12. Cytokine ELISA

For detection of cytokines, culture supernatants of spleen cells were collected after 48 hr stimulation and tested for the presence of the cytokines by antigen-capture ELISA using OptEIA Set Rat IFN-γ and IL-4 (BD Biosciences, San Diego, CA.). All assays were performed in triplicate. The concentration of IFN-γ and IL-4 in the culture supernatants was calculated by a linear-regression equation obtained from the absorbance values of the standards

13. Statistical analysis

Results were expressed as the mean ± SD. The statistical significances of the observed differences were as assessed by one way ANOVA. Data were analyzed by SPSS for Windows version 10.0 (SPSS Inc., Chicago, IL)

III. RESULTS

1. Sequence analysis of the cDNA encoding CsFABP and CsCP

The sequence analysis of the complete gene revealed that the 579 bp CsFABP cDNA (accession number AF527454), covers a single open reading frame that directs a 134 amino acid with a molecular mass of a 15.2 kDa (including the methionine residue, Fig. II-1 A). The Sequence analysis of this fragments revealed that the 1,053 bp *C. sinensis* cysteine proteinase (CsCP) covered one open reading frame that directs a 324-residues protein with molecular mass of a 37.6 kDa (Fig II-1 B). The CsFABP cDNA sequence displayed a significant homology to the schistosome cytosolic FABPs; it exhibited a 49% amino acid sequence identity/ 89% similarity to the *S. japonicum.* It also showed a high sequence similarity to the *S. mansoni* (Sm14) (83% pairwise amino acid sequence similarity in ORFs), and to the *F. hepatica* FABP (80% pairwise amino acid sequence similarity in ORFs). In addition, the sequence similarities with two mammalian proteins (the rat liver and rat intestinal FABP and human liver FABP), were 72%, 62% and 62% respectively (Fig. II-2 A). The CsCP sequence showed a high sequence similarity to the *C. sinensis* cysteine proteinase 1 (99 % pairwise amino acid sequence similarity in ORFs, AF093242) CsCP sequence displayed significant homology to the *Paragonimus westermani* pre-procathepsin L,

exhibiting 53% amino acid sequence identity and 80% similarity and also showed high sequence similarity to the *S. japonicum* cathepsin L1 (85% pairwise amino acid sequence similarity in ORFs), *S. mansoni*, and *F. hepatica* cysteine proteinase (73%, 68% pairwise amino acid sequence similarity in ORFs). In addition, the sequence similarity with three mammalian proteins, the rat cathepsin L, mouse cathepsin L, and human cathepsin L, were 70%, 64% and 64%, respectively (Fig. II-2 B).

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                                                  CCTCGTTCTCGCACATTTGTTCAGC
ATGTCGGCTTTCATCGGTAGTTGGAAGCTTTGTGAATCACAGAACTTTGATGAATTCTTG
 M  S  A  F  I  G  S  W  K  L  C  E  S  Q  N  F  D  E  F  L
AGAGAGCTCGGCGTCAACTTTTTCATTCGAAAAGCAGCGTCCACTTCGAAGCCAACTATA [120]
 R  E  L  G  V  N  F  F  I  R  K  A  A  S  T  S  K  P  T  I
ACTTTTGACAAGTTTGGGGACAATGGCTTGAAAATGAAGACGGAGACTATCCTCAAAACC [180]
 T  F  D  K  F  G  D  N  G  L  K  M  K  T  E  T  I  L  K  T
ACTGAGCAGTCGTTCACGTTCGGTGAGGAATTTGATGAGACAACGATTGATGGACGACAA [240]
 T  E  Q  S  F  T  F  G  E  E  F  D  E  T  T  I  D  G  R  Q
GTCAAGTCAACTGTAACCAGAGATTCTGACACCCAGTTGACTCAAGTACAAAAACACGAC [300]
 V  K  S  T  V  T  R  D  S  D  T  Q  L  T  Q  V  Q  K  H  D
GATGGTAACACTGTCATTGTCCGGAAAATTGAAGGAGATATGATGGTCACCACTGCAACC [360]
 D  G  N  T  V  I  V  R  K  I  E  G  D  M  M  V  T  T  A  T
TTCAAGAACATCACGTCTGTTCGGAAATACCAACGCCACTGATTCTTAGTTTTTTTATAC [402]
 F  K  N  I  T  S  V  R  K  Y  Q  R  H  *
AGTTCTTTATTATGTACACAGCCTGTCCACCCTTCTATCAGAATCGTCCAAGCTTTGATT
TACTCACCAAACACTGTTGAGCTATTCTCCTAACTGCGCTTATAATAAATATTTGTCTGG
CGAAAAAAAAAAA
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Figure II-1 A. Nucleotide and conceptual amino acid sequences of *C. sinensis* fatty acid binding protein (CsFABP). This sequence was submitted to Genbank under accession number AF527454.

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GCGACTTTTCGTGTGCTGCGTGTTGGTGACAACAATCTGGTCTGCTTTGGCCAGAACTACTCAAGTTGAG [70]
  R  L  F  V  C  C  V  L  V  T  T  I  W  S  A  L  A  R  T  T  Q  V  E
CCTGACAATGCCCGGGCACTATACGAGGAGTTCAAGCTGAAGTACAAGAAGACCTATTCAAATGATGATG [140]
 P  D  N  A  R  A  L  Y  E  E  F  K  L  K  Y  K  K  T  Y  S  N  D  D
ATGAGCTTCGATTCGAAATTTTCAAAGACAACTTGCTGCGCGCCAAGAGACTGCAGGAAATGGAACAGGG [210]
D  E  L  R  F  E  I  F  K  D  N  L  L  R  A  K  R  L  Q  E  M  E  Q  G
GACGGCGCAGTATGGTGTAACCCAATTTTCCGACCTGACCAGTGAGGAGTTCAAGACGCGCTATTTGAGG [280]
 T  A  Q  Y  G  V  T  Q  F  S  D  L  T  S  E  E  F  K  T  R  Y  L  R
ATGCGATTCGATGGGCCGATTGTCAGTGAGGATCTCACTCCAGAGGAGGATGTGACGATGGATAACGAGA [350]
 M  R  F  D  G  P  I  V  S  E  D  L  T  P  E  E  D  V  T  M  D  N  E
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K  F  D  W  R  E  H  G  A  V  G  P  V  L  D  Q  G  K  C  G  S  C  W  A
ATTTTCTGTGATTGGGAATGTCGTGGGTCAGTGGTTCCGTAAGACTGGGCATCTTCTAGCTCTCAGTGAA [490]
  F  S  V  I  G  N  V  V  G  Q  W  F  R  K  T  G  H  L  L  A  L  S  E
CAGCAACTCGTTGATTGCGACTATTTGGATGACGAATGTGATGGCGGTTATCCCCCACAGACGTACACTG [560]
Q  Q  L  V  D  C  D  Y  L  D  D  E  C  D  G  G  Y  P  P  Q  T  Y  T
CAATTCAAAAGATGGGCGGGTTGGAGCTGGCATCGGATTACCCCTACACCGGAGTTGGTGGAATATGTCA [630]
A  I  Q  K  M  G  G  L  E  L  A  S  D  Y  P  Y  T  G  V  G  G  I  C  H
TATGGACAAGTCGAAGTTCGTAGCCTATGTAAACGGTTCAACTATATTGCCTTTGAGCGAAAAGGTACAG [700]
  M  D  K  S  K  F  V  A  Y  V  N  G  S  T  I  L  P  L  S  E  K  V  Q
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 A  Q  K  L  R  A  I  G  P  L  S  S  A  L  N  A  D  T  L  Q  L  Y  K
GAGGAATCATGCGTCCCAAGTGGTGCGACCCGGCTGGAGTGAACCACGCTGTTCTCACCGTCGGCTACGG [840]
G  G  I  M  R  P  K  W  C  D  P  A  G  V  N  H  A  V  L  T  V  G  Y  G
CGTGCAGAACGGAAAGCCATACTGGATAGTGAAGAACAGTTGGGGCGAAGATTTTGGAGAAGAGGGCTAT [910]
  V  Q  N  G  K  P  Y  W  I  V  K  N  S  W  G  E  D  F  G  E  E  G  Y
TTCCGTATCTACCGAGGAGATGGAACCTGTGGAATCAACAGCATAGTTACTACAGCGATTATCAAATAGA [976]
 F  R  I  Y  R  G  D  G  T  C  G  I  N  S  I  V  T  T  A  I  I  K  *
CCGACTCCATGTTGGTATTTGACTCATACAGaCAATAAAAGCAAATTTTGCAAAAAAAAAAAAAAAAAAA
AAA
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Figure II-1 B. Nucleotide and conceptual amino acid sequences of *C. sinensis* cysteine proteinase (CsCP).

		βA	α I		α II	βB		βC	
CsFABP	MSAF	IGSWKLCESQ	NFDEFLREL	GVN	FFIRKAAST	SKPTITFDKF	GD	NGLKMKTET	IL
Sj-FABP	MSSF	LGKWKLSESH	NFDAVMSKL	GVS	WATRQIGNT	VTPTVTFTMD	GD	T-MTMLTES	TF
Sm14-FABP	MSSF	LGKWKLSESH	NFDAVMSKL	GVS	WATRQIGNT	VTPTVTFTMD	GD	K-MTMLTES	TF
Fh-FABP	MANF	VGSWKLEQSE	NMDAVLQKL	GIN	VIKRKLITS	SKPEITFTLE	GN	K-MTMKTVS	AL
Fg-FABP	----	--SWKYGDSE	NMEAYLKKL	GIS	SDMVDKILN	AKPEFTFTLE	GN	Q-MTIKMVS	SL
P2	SNKF	LGTWKLVSSE	NFDEYMKAL	GVG	LATRKLGNL	AKPRVIISKK	GD	I-ITIRTES	PF
rL-FABP	-MNF	SGKYQVQSQE	NFEPFMKAM	GLP	EDLIQKGKD	IKGVSEIVHE	GK	K-VKLTITY	GS
hL-FABP	-MSF	SGKYQLQSQE	NFEAFMKAI	GLP	EELIQKGKD	IKGVSEIVQN	GK	H-FKFTITA	GS
rI-FABP	-MAF	DGTWKVDRNE	NYEKFMEKM	GIN	VVKRKLGAH	DNLKLTITQE	GN	K-FTVKESS	NF

	βD		βE		βF		βG		βH	
CsFABP	KTTEQSFT	FGE	EFDETT	ID	GRQVKSTVTRD	SDT	QLTQVQKH	DD	G-NTVIV**RKIE**	**GD**
Sj-FABP	KNLSVTFK	FGE	EFDEKT	SD	GRSVKSVVTKD	SES	KITHTQKD	SK	N-TTVIVREIV	GD
Sm14-FABP	KNLSCTFK	FGE	EFDEKT	SD	GRNVKSVVEKN	SES	KLTQTQVD	PK	N-TTVIVREVD	GD
Fh-FABP	KTTVISFT	FGE	EFKEET	AD	GRTVMTTFTKD	SDS	KISQVQKC	PE	N-TTHVVREVT	GG
Fg-FABP	KTKITTFT	FGE	EFKEET	AD	GKTAMTTVTKD	SES	KMTQVTTG	PE	Y-TTHVVREVV	GD
P2	KNTEISFK	LGQ	EFEETT	AD	NRKTKSTVTLA	RGS	-LNQVQKW	NG	N-ETTIKRKLV	DG
rL-FABP	KVIHNEFT	LGE	ECELET	MT	GEKVKAVVKME	GDN	KMVTTFKG	IK	--S---VTEFN	GD
hL-FABP	KVIQNEFT	VGE	ECELET	MT	GEKVKTVVQLE	GDN	KLVTTFKN	IK	--S---VTELN	GD
rI-FABP	RNIDVVFE	LGV	DFAYSL	AD	GTELTGTWTME	GNK	LVGKFKRV	DN	GKELIAV**REIS**	**GN**

	βI		βJ		
CsFABP	MMVTTATF	KN	ITSVRKYQRH-	[133]	Identity/similarity
Sj-FABP	TMKTTVTV	DD	VTAIRNYKRL-	[132]	49%/89%
Sm14-FABP	TMKTTVTV	GD	VTAIRNYKRLS	[133]	49%/83%
Fh-FABP	KMIATVTV	GD	VKAVNNYHKV-	[132]	47%/80%
Fg-FABP	KMIATWTV	GD	VKAVTTLLKA-	[126]	35%/68%
P2	KMVVECKM	KD	VVCTRIYEKV-	[131]	41%/81%
rL-FABP	TITNTMTL	GD	IVYKRVSKRI-	[127]	30%/62%
hL-FABP	IITNTMTL	GD	IVFKRISKRI-	[127]	24%/62%
rI-FABP	ELIQTYTY	EG	VEAKRIFKKE-	[132]	23%/72%

Figure II-2 A. Multiple alignment of deduced amino acid sequences for mammalian, parasites, and CsFABP. Also shown are the secondary structures of the ten β strands, βA to βJ, and two α helices, αI and αII, which typical of cLBPs and based on the secondary structure as determined by three-dimentional crystallographic studies for bovine myelin P2 protein.[61] Sj-FABP, *S. japonicum* FABP [62,63]; Sm14-FABP, *S. mansoni* FABP[64]; Fh-FABP, *F .hepatica* FABP (CAB65015); Fg-FABP, *F. gigantica* FABP[65]; P2, bovine myeline protein; rBABP, rat bile acid binding protein[66]; rI-FABP, rat intestine-FABP[67]; rL-FABP, rat liver FABP[68]; hL-FABP, human liver FABP[69].

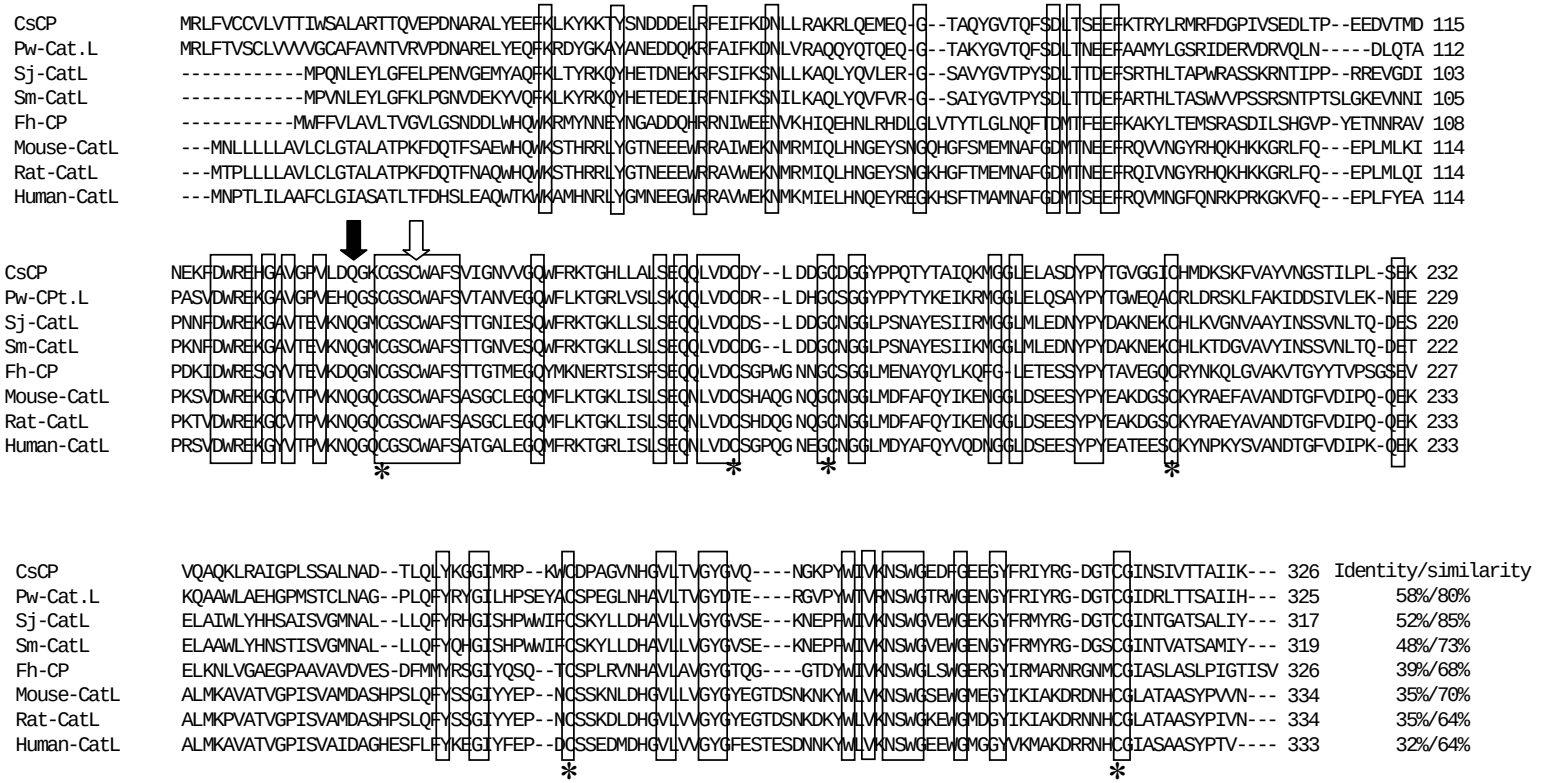

CsCP MRLFVCCVLVTTIWSALARTTQVEPDNARALYEEFKLKYKKTYSNDDDELRFEIFKDNLLRAKRLQEMEQ-G--TAQYGVTQFSDLTSEEFKTRYLRMRFDGPIVSEDLTP--EEDVTMD 115
Pw-Cat.L MRLFTVSCLVVVVGCAFAVNTVRVPDNARELYEQFKRDYGKAYANEDDQKRFAIFKDNLVRAQQYQTQEQ-G--TAKYGVTQFSDLTNEEFAAMYLGSRIDERVDRVQLN-----DLQTA 112
Sj-CatL ----------MPQNLEYLGFELPENVGEMYAQFKLTYRKQYHETDNEKRFSIFKSNLLKAQLYQVLER-G--SAVYGVTPYSDLTTDEFSRTHLTAPWRASSKRNTIPP--RREVGDI 103
Sm-CatL ----------MPVNLEYLGFKLPGNVDEKYVQFKLKYRKQYHETEDEIRFNIFKSNILKAQLYQVFVR-G--SAIYGVTPYSDLTTDEFARTHLTASWVPSSRSNTPTSLGKEVNNI 105
Fh-CP ----------MWFFVLAVLTVGVLGSNDDLWHQWKRMYNNEYNGADDQHRRNIWEENVKHIQEHNLRHDLGLVTYTLGLNQFTDMTFEEFKAKYLTEMSRASDILSHGVP-YETNNRAV 108
Mouse-CatL ---MNLLLLLAVLCLGTALATPKFDQTFSAEWHQWKSTHRRLYGTNEEEWRRAIWEKNMRMIQLHNGEYSNGQHGFSMEMNAFGDMTNEEFRQVVNGYRHQKHKKGRLFQ---EPLMLKI 114
Rat-CatL ---MTPLLLLAVLCLGTALATPKFDQTFNAQWHQWKSTHRRLYGTNEEEWRRAVWEKNMRMIQLHNGEYSNGKHGFTMEMNAFGDMTNEEFRQIVNGYRHQKHKKGRLFQ---EPLMLQI 114
Human-CatL ---MNPTLILAAFCLGIASATLTFDHSLEAQWTKWKAMHNRLYGMNEEGWRRAVWEKNMKMIELHNQEYREGKHSFTMAMNAFGDMTSEEFRQVMNGFQNRKPRKGKVFQ---EPLFYEA 114
CsCP NEKFDWREHGAVGPVLDQGKCGSCWAFSVIGNVVGQWFRKTGHLLALSEQQLVDCDY--LDDGCDGGYPPQTYTAIQKMGGLELASDYPYTGVGGICHMDKSKFVAYVNGSTILPL-SEK 232
Pw-CPt.L PASVDWREKGAVGPVEHQGSCGSCWAFSVTANVEGQWFLKTGRLVSLSKQQLVDCDR--LDHGCSGGYPPYTYKEIKRMGGLELQSAYPYTGWEQACRLDRSKLFAKIDDSIVLEK-NEE 229
Sj-CatL PNNFDWREKGAVTEVKNQGMCGSCWAFSTTGNIESQWFRKTGKLLSLSEQQLVDCDS--LDDGCNGGLPSNAYESIIRMGGLMLEDNYPYDAKNEKCHLKVGNVAAYINSSVNLTQ-DES 220
Sm-CatL PKNFDWREKGAVTEVKNQGMCGSCWAFSTTGNVESQWFRKTGKLLSLSEQQLVDCDG--LDDGCNGGLPSNAYESIIKMGGLMLEDNYPYDAKNEKCHLKTDGVAVYINSSVNLTQ-DET 222
Fh-CP PDKIDWRESGYVTEVKDQGNCGSCWAFSTTGTMEGQYMKNERTSISFSEQQLVDCSGPWGNNGCSGGLMENAYQYLKQFG-LETESSYPYTAVEGQCRYNKQLGVAKVTGYYTVPSGSEV 227
Mouse-CatL PKSVDWREKGCVTPVKNQGQCGSCWAFSASGCLEGQMFLKTGKLISLSEQNLVDCSHAQGNQGCNGGLMDFAFQYIKENGGLDSEESYPYEAKDGSCKYRAEFAVANDTGFVDIPQ-QEK 233
Rat-CatL PKTVDWREKGCVTPVKNQGQCGSCWAFSASGCLEGQMFLKTGKLISLSEQNLVDCSHDQGNQGCNGGLMDFAFQYIKENGGLDSEESYPYEAKDGSCKYRAEYAVANDTGFVDIPQ-QEK 233
Human-CatL PRSVDWREKGYVTPVKNQGQCGSCWAFSATGALEGQMFRKTGRLISLSEQNLVDCSGPQGNEGCNGGLMDYAFQYVQDNGGLDSEESYPYEATEESCKYNPKYSVANDTGFVDIPK-QEK 233
CsCP VQAQKLRAIGPLSSALNAD--TLQLYKGGIMRP--KWCDPAGVNHGVLTVGYGVQ----NGKPYWIIKNSWGEDFGEEGYFRIYRG-DGTCGINSIVTTAIIK--- 326 Identity/similarity
Pw-Cat.L KQAAWLAEHGPMSTCLNAG--PLQFYRYGILHPSEYACSPEGLNHAVLTVGYDTE----RGVPYWTVRNSWGTRWGENGYFRIYRG-DGTCGIDRLTTSAIIH--- 325 58%/80%
Sj-CatL ELAIWLYHHSAISVGMNAL--LLQFYRHGISHPWWIFCSKYLLDHAVLLVGYGVSE---KNEPFWIVKNSWGVEWGEKGYFRMYRG-DGTCGINTGATSALIY--- 317 52%/85%
Sm-CatL ELAAWLYHNSTISVGMNAL--LLQFYQHGISHPWWIFCSKYLLDHAVLLVGYGVSE---KNEPFWIVKNSWGVEWGENGYFRMYRG-DGSCGINTVATSAMIY--- 319 48%/73%
Fh-CP ELKNLVGAEGPAAVAVDVES-DFMMYRSGIYQSQ--TCSPLRVNHAVLAVGYGTQG----GTDYWIVKNSWGLSWGERGYIRMARNRGNMCGIASLASLPIGTISV 326 39%/68%
Mouse-CatL ALMKAVATVGPISVAMDASHPSLQFYSSGIYYEP--NCSSKNLDHGVLLVGYGYEGTDSNKNKYWLVKNSWGSEWGMEGYIKIAKDRDNHCGLATAASYPVVN--- 334 35%/70%
Rat-CatL ALMKPVATVGPISVAMDASHPSLQFYSSGIYYEP--NCSSKDLDHGVLVVGYGYEGTDSNKDKYWLVKNSWGKEWGMDGYIKIAKDRNNHCGLATAASYPIVN--- 334 35%/64%
Human-CatL ALMKAVATVGPISVAIDAGHESFLFYKEGIYFEP--DCSSEDMDHGVLVVGYGFESTESDNNKYWLVKNSWGEEWGMGGYVKMAKDRRNHCGIASAASYPTV---- 333 32%/64%

Figure II-2 B. Multiple alignment of deduced amino acid sequences for mammalian, parasites, and CsCP. CsCP, *Clonorchis sinensis* cysteine proteinase (AF093242); Pw-Cat L, *P. westermani* pre-procathepsin L (U70537); Sj-Cat L, *S. japonicum* cathepsin L (AF510740); Sm-Cat L, *S. mansoni* cathepsin L (Q26534); Fh-CP, *F .hepatica* cysteine protreinase (U62288); Rat-CatL, rat cathepsin L (NM_013156); Mouse-Cat L, mouse cathepsin L (NP-954599); Human -Cat L, human cathepsin L (NM_145918). Box areas indicate the identity from CsCP. Comparison of the cathepsin L-like protease alignment comparing amino acid sequencea spanning the active site which is labeled with a white arrow the predicted glutamine of the oxyanion hole which is marked with a filled arrow. *, indicate conserved cystein residues related with forming disulfide bridges.

2. Expression and purification of recombinant CsFABP and CsCP

cDNA of the FABP and cDNA of the CP from *C. sinensis* were respectively cloned into the expression vector pET28a and it was expressed in *E. coli.* The recombinant protein was purified by affinity chromatography (Fig. II-3A, B). Although a majority of rCsFABP was produced as insoluble aggregates, several hundred micrograms per liter (about 450 μg) of soluble protein were also obtained. The recombinant fusion protein was efficiently eluted as a 22 kDa molecule. The increase in molecular weight of the molecule is due to the 56 amino acids at the N –terminus of the recombinant protein. The recombinant fusion protein CsCP was efficiently eluted as 41 kDa molecules. The increase in molecular weight is due to the 34 amino acid at N –terminus of the recombinant protein.

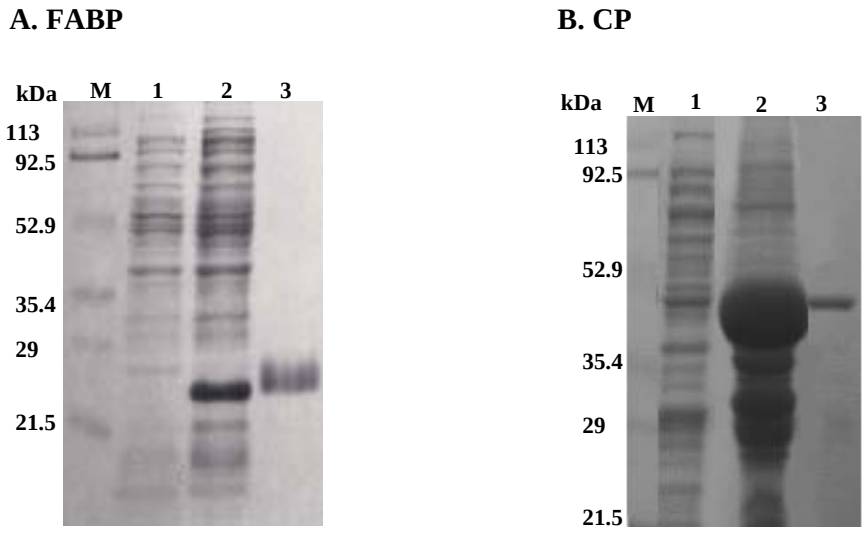
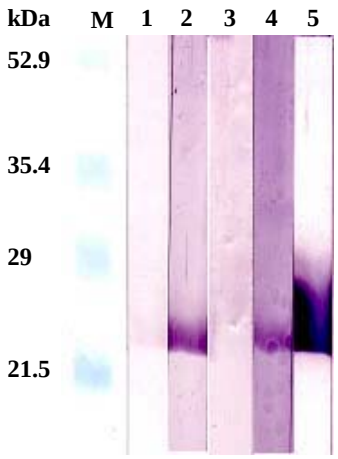


Figure II-3. Recombinant proteins purified by affinity Ni^{2+}–NTA column chromatography. The proteins were viewed on Coomassie blue stained 12% SDS-PAGE. Lane 1, the total cell fraction of non-induced condition after bacterial lysis with lysozyme; lane 2, the fraction of 1 mM IPTG induction; 1ane 3, the elution fraction.

3. Antigenicity of rCsFABP and rCsCP

The rCsFABP or rCsCP was evaluated for its antigenicity to the helminth by the western blotting method as was performed and described in the Materials and Methods section. The rCsFABP and rCsCP were strongly reacted with the sera from patients that were serologically proven to have the diagnosis of clonorchiasis and sera from rat with infected *C. sinensis* (Fig. II-4 A, B).

A. FABP



B. CP

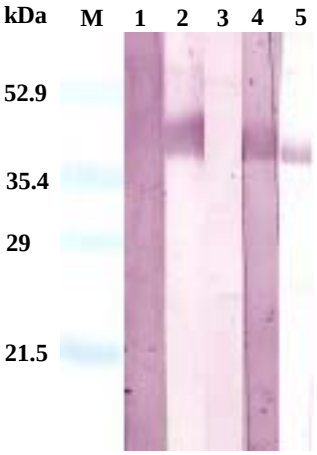
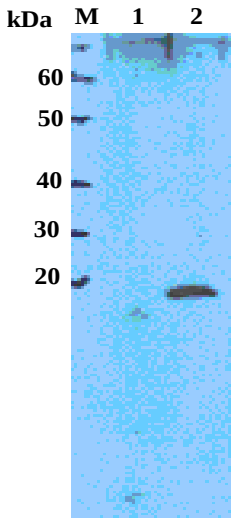


Figure II-4. Antigenicity of recombinant protein by western blot analysis. Lane 1, human control sera; lane 2, sera from patients with serologically proven clonorchiasis; lane 3, sera from uninfected; lane 4, sera from rat with infected *C. sinensis*; lane 5, sera from rat with immunized recombinant protein

4. DNA construction and expression in vitro

The full-length cDNA of CsFABP or CsCP was cloned into the mammalian expression vector pcDNA3.1 (+), for generating the pcDNA3.1-FABP or pcDNA3.1-CP. Expression of the inserted gene is driven by the human CMV promoter/enhancer. The cloned CsFABP cDNA sequence was verified by sequencing and showed 96% identity to the published nucleotide sequence. Prior to immunization of animals, the DNA vaccine construct was tested in vitro for the expression of protein. Plasmid DNA was purified and delivered into HEK 293 cells by liposome–mediated transfection. As shown in Fig. II-5 A, pcDNA3.1-FABP is able to direct the production of an immunoreactive 15.2 kDa protein within the cellular compartment. The western blot of each extract after probing with anti–CsCP sera is shown in Fig. II-5 B. As can be seen pcDNA3.1-CsCP is able to direct the production of an immunoreactive 36.7 kDa protein within the cellular compartment. The empty vector pcDNA3.1 was included as negative control.

A. FABP



B. CP

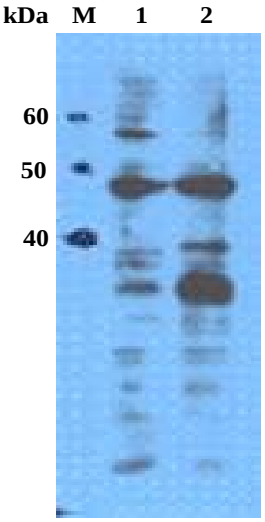
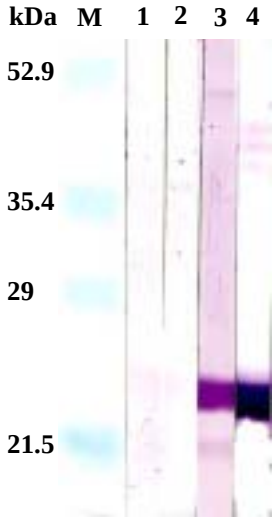


Figure II-5. *In vitro* expression of DNA constructs in HEK 293 cells. Extract from transfected HEK 293 cells were electrophoresised, transferred to nitrocellulose and probed with rat serum against rCsFABP or rCsCP. Lane 1, HEK 293 cells were transfected with pcDNA3.1; lane 2, pcDNA3.1-FABP or pcDNA 3.1-CP.

5. Expression of DNA construction *in vivo*

Next we investigated the potential of the pcDNA3.1-FABP or pcDNA3.1-CP expression construct to induce an antibody response *in vivo*. Sera from vaccinated rats were analyzed in western blot with purified rCsFABP or rCsCP (Fig. II-6 A, B). The production of specific antibodies in pcDNA3.1-FABP or pcDNA3.1-CP immunized rats further confirmed that pcDNA3.1-FABP or pcDNA3.1-CP was expressed and induced significant immune response in immunized animals.

A. FABP



B. CP

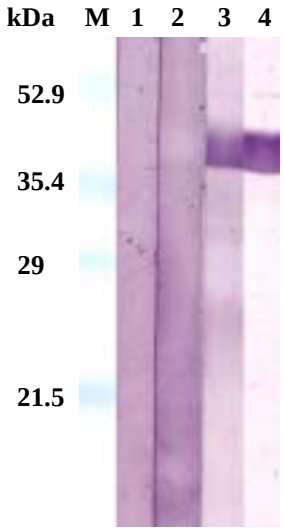
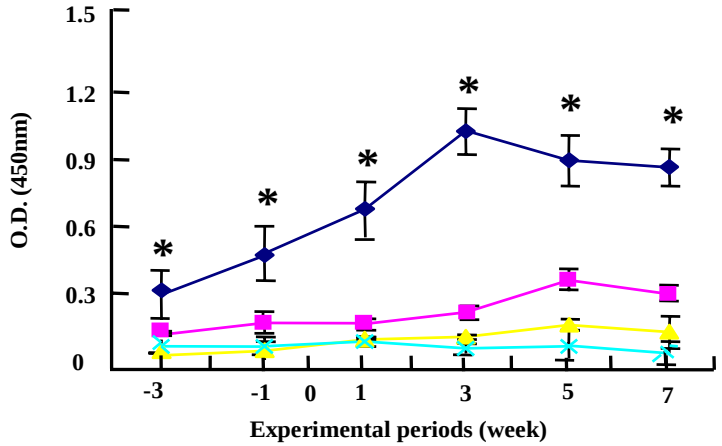


Figure II-6. Western blot analysis of recombinant protein against DNA vaccinated rat sera. Rats were intradermally immunized with 200μg pcDNA3.1-FABP or pcDNA3.1-CP or as a control, the empty vector pcDNA3.1. Lane 1, healthy rat sera; lane 2, vaccinated sera with pcDNA3.1; lane 3, vaccinated sera with pcDNA3.1-FABP or pcDNA3.1-CP; lane 4, immunized sera with recombinant protein

6. Specific IgG antibody responses in vaccinated rat sera

SD rats were immunized by two intradermal injections of 200 μg pcDNA3.1-FABP, pcDNA3.1-CP, pcDNA3.1, or PBS at 2 weeks intervals. Immunized rats infected with 50 *C. sinensis* metacercariae at 3 weeks post the last DNA injection. Levels of rCsFABP or rCsCP specific IgG antibodies were evaluated by ELISA on sera from pcDNA3.1-FABP, pcDNA3.1-CP, pcDNA3.1-immunized, or PBS-immunized animals. The specific IgG response was strongly induced in all intradermally immunized rats with pcDNA3-FABP or pcDNA3.1-CP, where as weakly specific IgG were detected in control animals (pcDNA3.1 or PBS group). The antibody response increased rapidly to a peak 8 weeks (3 weeks post infection) and persisted at a constant level until 12 weeks (7 weeks post infection) (Fig. II-7). In particular, oral infection with metacercariae induced a strong boosting effect on the antibody response elicited by genetic immunization.

A. FABP



B. CP

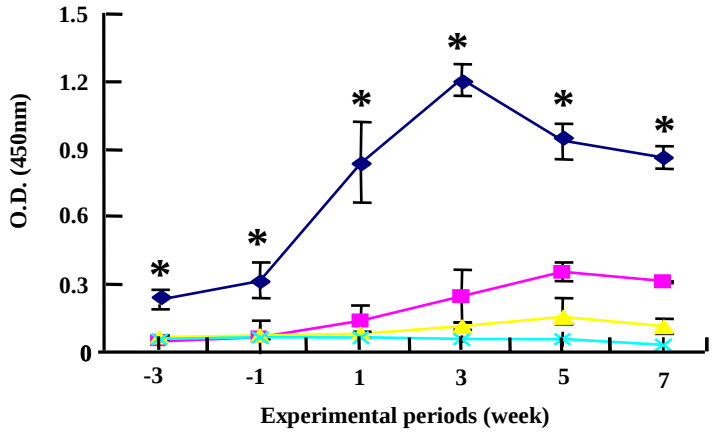
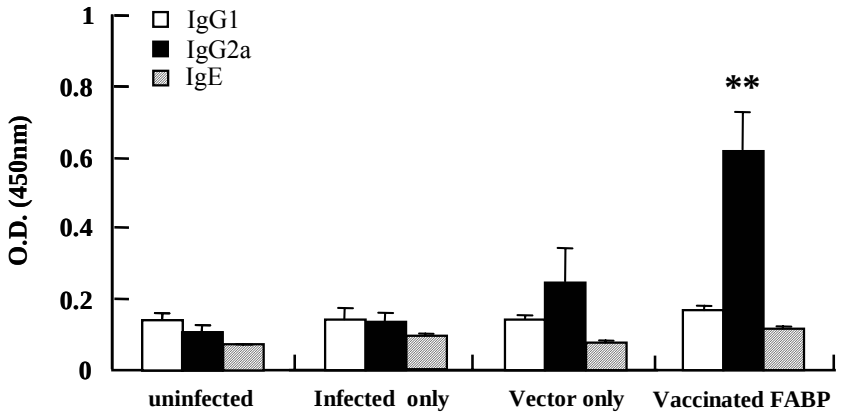


Figure II-7. ELISA analysis of specific IgG antibody response in vaccinated rats sera. × , uninfected sera; ▲ , infected sera; ■, vaccinated sera with pcDNA3.1; ◆, vaccinated sera with pcDNA3.1-FABP or pcDNA3.1-CP; *, statistically significant differences compared to pcDNA3.1 group ($p<0.05$)

7. Specific production of IgG2a in vaccinated rat sera

As shown in Fig. II-8 A, rats vaccinated with pcDNA3.1-FABP group developed an effective immune response showing substantially higher levels of specific IgG2a antibody compared with control rats (pcDNA3.1 or PBS group) at 7 weeks post infection. Total serum IgG1, IgE were not found to be similar in all groups of rats regardless of disease outcome. Vaccinated CP group with pcDNA3.1-CP in rats that developed an effective immune response had substantially higher levels of pcDNA3.1-CsCP group-specific IgG2a antibody titer compared with control group rats (Fig. II-8 B). The level of IgG1 was also significant compared with the control animals. Finally, IgE was not found to be similar in all groups of rats regardless of disease outcome.

A. FABP



B. CP

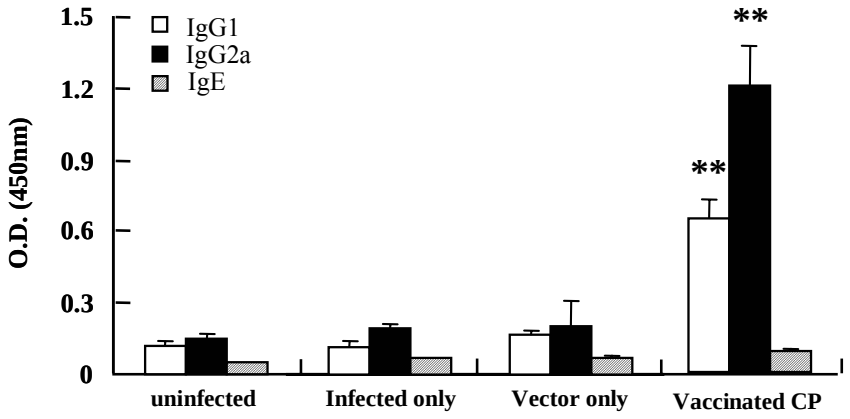
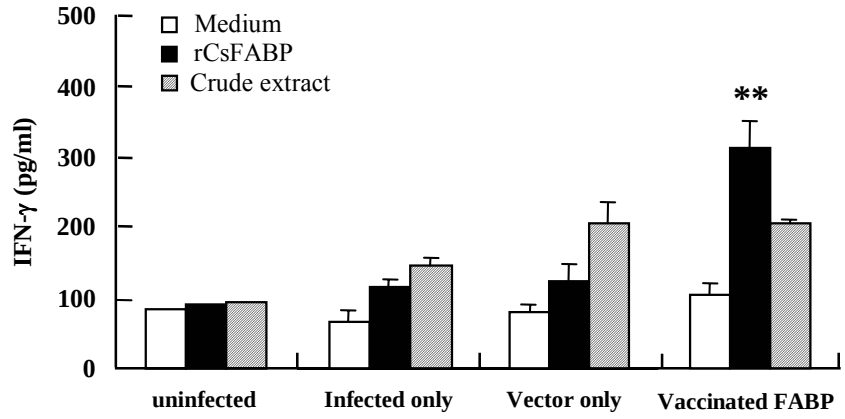


Figure II-8. ELISA analysis of specific IgG1, IgG2a, and IgE production in sera of vaccinated rats at post 7 weeks infection. **, statistically significant differences compared to vector only groups ($p<0.001$)

8. Specific production of IFN- γ in vaccinated rat

To examine the cell-mediated immune response to rCsFABP and crude extract of *C. sinensis*, cytokine profile of spleen cells were determined from rats immunized with pcDNA3.1-FABP, pcDNA3.1, and PBS. With respect to cytokine profile, supernatants of spleen cell cultures from vaccinated FABP group contained high levels of IFN-γ compared to the negative control groups (the vector only and infected only group). The rCsFABP protein significantly induced the production of IFN-γ in spleen cells from pcDNA-FABP immunized mice ($p < 0.001$ in both groups) (Fig. II-9 A), and IL-4 was not detected in all groups (Fig. II-10A). With respect to cytokine profile, supernatants of spleen cell cultures from pcDNA3.1-CsCP immunized animals contained high levels of IFN- γ compared to the negative control groups. The rCsCP and crude extract *C. sinensis* proteins significantly induced the production of IFN- γ in cells from pcDNA3.1-CsCP-immunized mice ($p < 0.001$ in both groups) (Fig. II-9 B), and IL-4 was not detected in all groups (Fig. II- 10 B).

A. FABP



B. CP

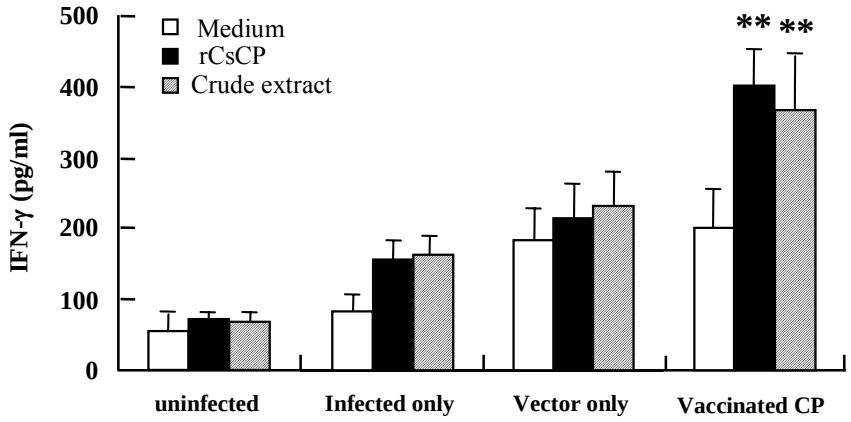
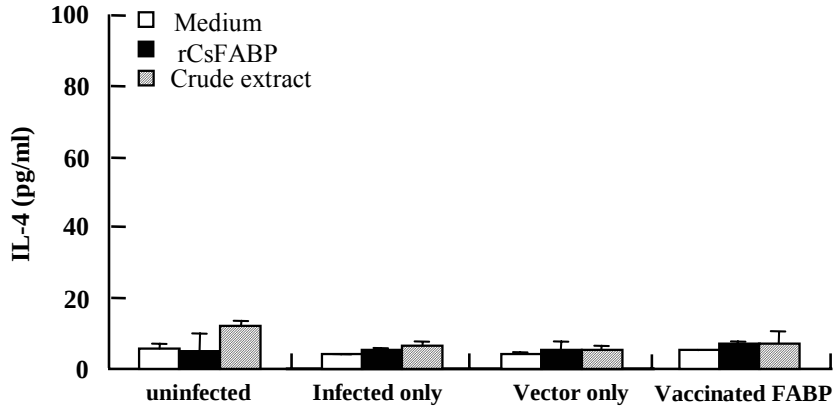


Figure II-9. ELISA analysis of IFN-γ secretion by splenocytes upon *in vitro* stimulation with different antigens in vaccinated rats at post 7 weeks infection. **, statistically significant differences compared to vector only groups ($p<0.001$)

A. FABP



B. CP

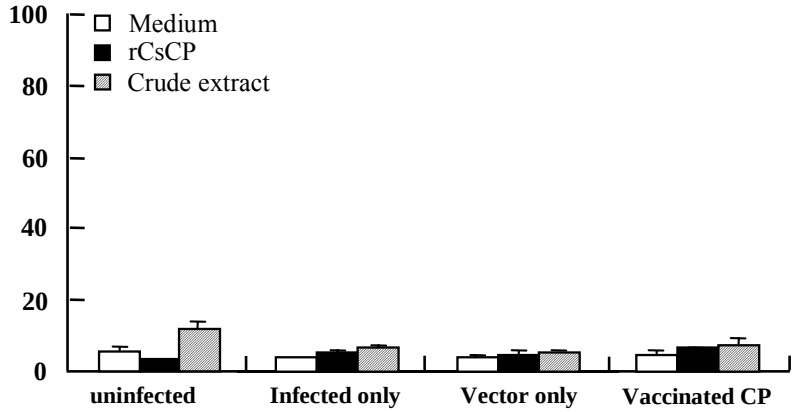


Figure II-10. ELISA analysis of IL-4 secretion by splenocytes upon *in vitro* stimulation with different antigens in vaccinated rats at post 7 weeks infection.

9. Efficacy of DNA vaccination generating protective immunity against *C. sinensis*

Rats were sacrificed at 49 days (7 weeks) after the challenge infection, and the data related with the effect of the DNA vaccine on rat pathology and parasite development are summarized in Table II-2. Spleen and liver enlargement were increased in the group of pcDNA3.1-FABP. The recovered adult worm numbers were significantly reduced in vaccinated FABP group (40.9%, $p<0.05$) and the vaccinated CP group (31.5%, $p<0.05$). The the EPG seemed significantly reduced in vaccinated FABP group (27.5%, $p<0.05$) and the vaccinated CP group (15.7%, $p<0.05$). But, the fecundity of the parasites (EPG/ Number of recovered adult worms after sacrifice) seemed not reduced significantly. The eggs in the stool specimens were first demonstrated 22th day after infection of pcDNA3.1-FABP group, and 26th day from those of other groups. The egg-laying capacity of *C. sinensis* in all groups seemed fairly good and relatively stable 39th day after infection (Table II-3). Taken together, the pcDNA3.1-FABP vaccination did not affect fecundity of the adult worms but made worm burdens reduced.

Table II-2. Pathology, worms and egg loads in DNA vaccinated rats with at 7 week after infection.

		DNA vaccinated [a]		
	Infected	**Vector only**	**FABP**	**CP**
No. of rats	5	5	5	5
Rat body weight (gm)	269.5 ± 12.8 [b]	269.5 ± 12.8	263.8 ± 8.2	256.6 ± 15.7
Spleen (gm)	0.63 ± 0.06	0.64 ± 0.02	0.73 ± 0.04 (+15.8%, $p<0.05$) [c]	0.67 ± 0.06
Liver (gm)	8.1 ± 0.4	8.3 ± 0.4	8.8 ± 0.4	8.7 ± 0.6
No. of adult worms	34.2± 5.2	35.4 ± 2.7	20.2 ± 5.1 (-40.9%, $p<0.05$)	23.4 ± 7.4 (-31.5%, $p<0.05$)
No. of eggs per gram of rat feces (x100)	10.2 ± 0.9	9.9 ± 1.3	7.4 ± 1.1 (-27.5%, $p<0.05$)	8.6 ± 0.5 (-15.7%, $p<0.05$)

[a], Rats were vaccinated two times with the empty vector pcDNA3.1, pcDNA3.1-FABP, or pcDNA3.1-CP, then orally administered with 50 *C. sinensis* metacercariae and sacrificed 7 weeks later.

[b], Mean± S.D.

[c], Statistically significant the decrease compare with the vector only group.

Table II-3. Eggs per gram of rat feces (EPG) during experimental infection periods.

Days after experimental infection	Mean EPG by group (X100)			
	Infected	Vector only	FABP	CP
19	-	-	-	-
22	-	-	0.16 ± 0.16 [b]	-
26	1.28 ± 0.30	0.48 ± 0.20	0.56 ± 0.20	0.16 ± 0.08
29	9.04 ± 1.36	5.04 ± 0.84	4.56 ± 0.84	5.04 ± 0.60
33	14.28 ± 1.36	8.6 ± 1.04	9.76 ± 0.64	8.96 ± 1.44
36	15.8 ± 1.88	15.68 ± 1.40	12.16 ± 0.08	13.68 ± 1.32
39	20.64 ± 1.76	18.8 ± 2.28	16.6 ± 1.36	17.76 ± 0.88
41	14.9 ± 1.00	15.04 ± 0.92	10.32 ± 1.76	14.68 ± 1.04
45	12.32 ± 0.48	11.8 ± 1.72	9.04 ± 0.52	10.2 ± 0.76
49	10.16 ± 0.88	9.92 ± 1.32	7.36 ± 1.08 (-27.5%, $p<0.05$) [c]	8.56 ± 0.48 (-15.7%, $p>0.05$) [c]

[a], Rats were vaccinated two times with PBS, the empty vector pcDNA3.1, pcDNA3.1-FABP, or pcDNA3.1-CP, then orally administered with 50 *C. sinensis* metacercariae. Thereafter, stool examination was performed to demonstrate *C. sinensis* eggs by formalin-ether sedimentation technique until 49days of infection.

[b], Mean± S.D.

[c], Statistically significant the decrease compare with the vector only group.

Discussion

In this study, the cloning, expression of recombinant CsFABP and CsCP, and the DNA vaccination technology applied for clonorchiasis in the laboratory animals, and the results showed that i.d. immunization of SD rats with naked DNA encoding for CsFABP or CsCP induces a Th1 type immune response and reduction of worm burdens.

Immunization with DNA thus mimics aspects of live infection, with pathogen proteins synthesized endogenously by host cells. This synthesis leads to the induction of a cytotoxic T lymphocyte (CTL) via the major histocompatibility complex (MHC) class I- restricted pathway. Concurrently, proteins are released extracellularly. It is believed that this exogenously released antigen primes the induction of a humoral response, as well as a helper T lymphocyte (Th) response via MHC class II- restricted antigen presentation by antigen-presenting cells (APCs) that have taken up the foreign antigens. The mechanisms of action of DNA vaccines have not been entirely elucidated yet. It has been hypothesized that DNA delivered to the target site is expressed in given tissue cells, and resulting antigen is picked up by bone-marrow derived cells which in turn transport it to lymphoid tissue, where antigen presentation occurs. Effector and memory B and T cells are formed and consequently, immune responses take place.[70] Generally, DNA vaccination induces Th-1 dependent rather than Th-2 dependent

immune response. This feature may be especially advantageous in immune prophylactics of parasitic disease, during which the Th-2 type response causes immunopathological reaction, including allergies and liver granuloma formation in the course of schistosomiasis. Preferential induction of Th-1 type response after intradermal injection of DNA may depend on secretion of IL-12 by APC present in the skin, which in turn induces IFN-γ directing a reponse toward Th-1type.[71] The mechanisms of action of DNA vaccines have been mainly investigated in mice using either the i.d. or i.m. routes of immunization. I.m. injection results in low-level transfection of myocytes whereas i.d. injection may directly transfect APCs.[72] Because myocytes express MHC class I at a low levels and do not constitutively express MHC class II or costimulatory molecules such as B7, and thus are unlikely to functional as effective APCs.[73] Skin is known to contain a relatively high proportion of Langerhans cells, but appears less likely in intra muscular immunization, as dendritic cells and macrophages are thought to be scarce in muscle.[74]

Fatty acid binding proteins are thought to play an important role in the intracellular transport of long chain fatty acids and their acyl-CoA ester.[75] Both metabolites are highly abundant within the bile ducts of the liver, which is where the adult liver flukes reside. Trematodes lack the enzymes for de novo biosynthesis of fatty acids and must obtain them from their

host.[76] Thus, fatty acid binding proteins are essential for survival and an immune response against them may represent parasite lethality and host immunoprotection. Although FABP has drawn great interest for being considered as a vaccine candidate, FABP of *C. sinensis* (CsFABP) has remained a relatively unknown experimental item.

A comparison of the deduced amino acid sequence of CsFABP with that of the published FABP sequence revealed that the protein has a significant conservation to the FABP/P2/CRABP/CRBP family of cytosolic lipid binding proteins. The cytosolic or intracellular FABPs are small proteins of between 13 and 16kDa in molecular size and they consist of 127-137 amino acids. They are composed of ten antiparallel β-strands that form a cytoplasmic FABP barrel and their acyl CoA ester in a non-covalent fashion.[42,77]

The sequence analysis revealed that the CsFABP contained a single open reading frame directing a 134 amino acid with an estimated molecular mass of a 15.2 kDa (Fig. II-1 A). As shown in Fig. II-2 A, it was found that a bold style of amino acids is highly conserved in CsFABP, that is, in the amino acids 108-113. This region is involved in substrate binding, as was proved by the observation that mutation in the rIFABP at Arg 106 (Arg 107 in the Cs FABP sequence) reduces the affinity for oleate 20 fold.[78] It was suggested that *C. sinensis* adult worms require a FABP with the capacity to bind with bile acid; this might aid the worm's ability to reside in bile ducts. However, this

hypothesis will require some further studies.

The liver fluke releases powerful proteolytic enzymes which are predominantly cysteine proteases.[50,79] Several experimental results indicate that the cysteine proteases may be a source of protective antigens.[51,53] Cysteine proteinase was regulated to the development activity of *C. sinensis* in the life cycle. Cysteine proteinase activity in acidic extracts of various developmental stages of *C. sinensis* (metacercariae, 1-, 2-, and 3-month old worms) was reported.[80] All the activities were maximum at acidic pH and showed inhibitor susceptibilities similar to the vertebrate cysteine proteinases. A soluble cysteine proteinase with a native molecular weight of approximately 20,000 +/- 1414 was partially purified from 1-, 2-, and 3-month worms. The molecular weight of similar activity in metacercariae was approximately 32,000. The intramuscular immunization of rats with the CPcDNA induced a high level of protection against infection by mc of the fluke[37]and the experiments suggested that after challenge infection, in the DNA vaccinated rats, the juvenile *F. hepatica* might be killed during penetration through the intestinal wall. CsCP cDNA sequence displayed significant homology to the mammalian trematodes capthepsin L. As shown Fig. II-2 B., rCsCP was and highly similarity to the *P. westermani* pre-procathepsin L in alingnment. These previous reported that cysteine proteinase of *P. westermani* and *C. sinensis* was belonging to the cruzipain-like group.[81] But, this result will be need for

further study. Cruzipain has been shown to be antigenic in *T. cruzi*-infected persons. The majority of Chagas' disease patients have serum antibodies reactive with cruzipain.[82,83] In addition, T-cell lines generated from *T. cruzi*-infected individuals have been shown to proliferate and produce IFN-γ, but not IL-4, after in vitro stimulation with cruzipain[84], indicating that cruzipain induces human Th1 cell responses. The fact that these cruzipain-specific T cells have a Th1-like cytokine profile is important because in mice, Th1 responses have been associated with resistance to *T. cruzi* infection, while Th2 responses have been associated with susceptibility.[85,86]

The recombinant CsFABP and CsCP reacted strongly to the sera from patients with serologically proven condition of chlonorchiasis and infected rat sera with *C. sinensis* adults (Fig. II-4 A, B). These results indicated that rCsFABP or CsCP is respectively a strong antigenicity from their host. Several reports have demonstrated the efficacy of FABP preparations when used as vaccines against trematode infections. Sm 14 (the *S. mansoni* homologue of SjFABP) has stimulated considerable interest as a vaccine candidate not only against *S. mansoni*, but also as a cross-species vaccine component against other flatworm infections, notably animal and human fascioliasis caused by *F. hepatica*.[28] FABP would be a promising candidate molecule for a molecular vaccine because trematodes use this protein for obtaining fatty acid from their host. Also, Cysteine proteinase has been

already shown their protectivity capacity against trematodes[54,55,87]; however equivalent proteinase from *C. sinensis* has not been tested to date by DNA vaccination.

CsFABP or CsCP was for the first time successfully expressed in mammalian cells both *in vitro* and *in vivo*. We transiently transfected HEK 293 cells pcDNA3.1-FABP or pcDNA3.1-CP and expressed in rat with the pcDNA3.1-FABP or pcDNA3.1-CP (Fig. II-5, 6)

Immunization into shaved back skin with pcDNA3.1-FABP or pcDNA3.1-CP induced elicited specific humoral and cellular immune response in SD rats. Two weeks after the first immunization, we found specific IgG in rats immunized the plasmid pcDNA3.1-FABPor pcDNA3.1-CP. By the end of the experiment this response was four times higher. This may be related to the factors of antibody production, including the amount of CsFABP or CsCP expression, development of a preferential CMI or the structure of the FABPor CP. Also, the strong and rapid boost effect observed after the infestation of rats with metacercariae indicates that native CsFABP or CsCP reactivated the specific antibody response induced by intradermal DNA immunization. The induction of a T-cell immune response after pcDNA3.1-FABP or pcDNA3.1-CP vaccination was evaluated by measuring cytokine production after in vitro stimulation of spleen cells with purified rCsFABP, rCsCP, or *C. sinensis* crude extract. The rCsFABP induced a high level of IFN-γ and was not detected Il-4

in the supernatant of splenocytes culture of vaccinated FABP group (Fig. II- 8, 9). The vaccinated CP group produced a high level of IFN-γ by rCsCP or *C. sinensis* crude extract in the supernatant of splenocytes culture, but was not secrestion of IL-4. These results indicated that immunization with the pcDNA3.1-FABP or pcDNA3.1-CP plasmid induces a Th1 cellular response. The predominance of IgG2a over IgG1 also supported this conclusion. In the schistosoma infected rat model, there is some evidence that antibodies of the IgG2a subclass are related to protection. Indeed a monoclonal IgG2a antibody directed against a *S. mansoni* 38kDa antigen has been showed to mediate ADCC and to protect against a challenge infection after passive transfer.[88] Also, ADCC activity appeared to build up during the time after DNA immunization with Sm28GST, following a slight increase in IgG2a titrer and a progressive decrease in IgG2b titers which indicates that specific IgG2a antibodies would be directed involved in this acivity.[89] As ADCC may be considered as major defense mechanism against *C. sinensis*, the DNA immunization approach we used might promote protective effect. However, this hypothesis will require some further studies.

Intradermal gene vaccination of mice activities antigen-specific T helper type 1 (Th1) cells that secrete high levels IFN-γ and a stimulate production of antibody of the IgG2a isotype. [90-92] IgG2a antibody formation is dependent on IFN-γ as an IgM- to- IgG2a switch factor and and is believed to typical for

Th1 response.[93,94] In contrast, IgG1 and particularly IgE antibody production were dependent on IL-4 secreted by Th2 cells.[95] But, The vaccinated CP group with pcDNA3-CsCP resulted in an increase of the IgG1 antibody whereas is it caused a non-increase IgE antibody formation. Although IgG1 antibody production, like as IgE production, is typical for a Th-2 cell response,[71] IgG1 formation is much less dependent on, or in some instances even independent of, the requirement for IL-4. Injection of anti-IL-4 antibodies inhibits IgE but not IgG1 formation in mice.[96]

Our report is the first to investigate the vaccine efficacy of the fatty acid binding protein and cysteine proteinase of *C. sinensis*. Reduced worm burdens and EPG against 50 metacercariae of *C. sinensis* infection in pcDNA3.1-FABP or pcDNA3.1-CP vaccinated rats at 7 weeks post infection.

Generally in rats, the infected metacercariae of *C. sinensis* excyst in the duodenum, and juvenile worms crawl through the ampulla and extrahepatic bile duct and arrive in the intrahepatic bile duct. Two to Three weeks after infection, the juvenile worms descend to the extrahepatic bile duct and mature to adults. Most of the adult worms reside at the bile duct confluence and at the proximal extrahepatic bile duct.[97, 98] It appears that *Fasciola* infection in cattle, a Th2 like IgG1 humoral response is counterproductive and leads to chronic infection.[99] There was a strong positive correlation between the numbers of fluke recovered and the IgG2a titer.[100] Moreover, these data suggested that

protective immune responses may be of the Th 1 type involving IFN-γ-activated macrophages and CTL. Although the metacercariae migration patterns in final host of *C. sinensis* may differ from those of *F. hepatica*, the mechanism by which the vaccines described elicit the protective effects remains unclear.

Further studies need to examine the role of different T-cell types in the protection and defense mechanisms of host induced by vaccination with pcDNA3.1-FABPor pcDNA3.1-CP.

CONCLUSIONS

The present EST analysis proved to be an effective tool for examining gene expression and identified several important genes for increasing and complementing our knowledge of the biology of *C. sinensis*. The dataset included the genes encoding the proteases, a lipid binding protein, the antigen proteins and the other genes of interest from a diagnostics, drug or vaccine development viewpoint. FABP and cysteine proteinase were selected DNA vaccine candidates by EST analysis.

The recombinant CsFABP or CsCP was induced antigenicity in human and rat infected with *C. sinensis*. Animals injected with pcDNA3.1-FABP or pcDNA3.1-CP developed specific antibodies which exhibited a dominance of IgG2a in sera. The DNA vaccine elicited the production of IFN-γ, but not IL-4 in splenocytes, suggesting the induction of a typical Th-1 dominated immune response in rats. FABP and CsCP may be a good candidate for use in future studies of vaccination against clonorchiasis.

Collectively, this is the first report of the genetic vaccination approach of *C. sinensis*. The pcDNA3.1-FABP or pcDNA3.1-CP leads to the elicitation of both antibody and CMI response of Th1 type, and confers protection against an infectious challenge.

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ABSTRACT IN KOREAN

간흡충의 유전자 분석 및 간흡충증에 대한 DNA 백신 개발

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간흡충은 담관에 기생하는 흡충류로 우리나라를 비롯하여 대만, 중국 남부, 월남 등에 유행하고 있다. 이 지역에 거주하는 주민 약 700만 명이 간흡충에 감염되어 있는 것으로 보이며 사람들은 피낭유충이 들어 있는 민물고기를 생식하거나 부당하게 조리하여 먹음으로써 간흡충에 감염된다. 간흡충 성충의 유전자 분석은 새로운 약재 개발과 백신 개발에 있어서 유용하다. 간흡충 성충의 cDNA library를 제작하고 무작위로 450개의 클론을 선택, 정제하고 난 뒤 각 염기 서열을 분석한 결과 415개의 클론을 얻을 수 있었다. Basic local alignment search tool (BLAST) programs를 사용하여 National center for biotechnology information (NCBI)에 있는 non-redundant database에서 각 EST의 유사성을 탐색하여 415개의 클론들 중 277개의 클론들이 이미 동정된 유전자에 높은 유사성을 보였고 이 중 220개는 7가지 기능으로 분류할 수 있었다. 나머지 138개는 어떠한 결과에도

일치를 보이지 않았다. 전체 결과를 통해 단백 분해 효소, 지질 결합 단백질, 항원 단백질 외에 진단, 약재, 백신 개발에 유용한 유전자들을 포함하고 있었다. 이러한 EST 분석을 통해 EST가 유전자 발현 분석에 유용한 기술이며 간흡충의 생물학적 특성을 폭넓고 깊이 있게 이해 할 수 있는 다양한 유전자들을 규명할 수 있었다. 이들 다양한 유전자들 중에서 지방산 결합 단백질은 숙주로부터 흡충의 대사에 필요한 긴 사슬 지방산을 세포 내로 전달하는 중요한 역할을 하는 것으로 알려져 있다.

지방산 결합 단백질은 이미 백신 후보 유전자로 알려져 있지만 간흡충에 대한 지방산 결합 단백질에 대한 연구는 없었다. 간흡충의 시스테인 단백 분해 효소는 담도 상피 세포 파괴 및 유충의 이동에 관여하며 병적인 변화를 초래하는 치명적인 요인이다. 간흡충의 지방산 결합 단백질과 시스테인 단백 분해 효소를 DNA 백신을 통해 면역성과 방어 효과에 관한 연구를 실시하였다. 간흡충 지방산 결합단백질과 시스테인 단백분해효소의 cDNA를 발현 벡터 pET28a 에 클로닝 시킨 뒤 대장균에서 과발현 시킨 후, Ni-nitrotriacetic acid (NTA) agarose resin를 사용한 친화크로마토크래피의 방법으로 분리하였다. 간흡충에 감염된 사람의 혈청과 쥐의 혈청에 대해 각각 재조합 지방산 결합 단백질과 시스테인 단백 분해 효소에 대한 항원성을 보였고 이를 통해 간흡충증에 대한 DNA 백신 후보자로의 가능성을 추론할 수 있었다. pcDNA3.1 벡터

에 지방산 결합 단백질과 시스테인 단백 분해 효소 cDNA를 클로닝 시킨 뒤 대장균에서 과발현 시킨 후 다량의 DNA를 얻은 후 이것을 가지고 각각 간흡충에서는 처음으로 시험관 내와 생체 안에서의 발현을 확인하였다. 지방산 결합 단백질과 시스테인 단백 분해 효소에 대한 DNA 백신을 실시한 쥐에 간흡충의 피낭유충을 감염시킨 뒤 얻은 혈청에 대해 각각 재조합 단백질에 대한 IgG2a 항체를 생성하였고 DNA 백신을 실시한 쥐의 비장을 통해 얻은 비장 세포에서는 IL-4 보다는 IFN-γ 의 유의한 증가를 보였다. 이를 통해 Th-2 보다는 Th-1에 우세한 면역 반응을 보였다. 또한, 지방산 결합 단백질 DNA 백신을 실시한 쥐에서의 충체 회수율은 40.9% ($p<0.05$) 감소를 보였고 시스테인 단백분해효소에 대한 DNA 백신을 실시한 쥐에서는 31.5% ($p<0.05$) 감소를 보였다. 이 결과를 통해 간흡충의 지방산 결합 단백질과 시스테인 단백 분해 효소의 각각의 DNA는 실험 동물인 쥐에 피내 접종을 통해 체액성, 세포 매개성 면역을 유도할 수 있었고 차후 간흡충증에 대한 간흡충의 백신 후보자로써의 가능성을 제시하였다.

핵심되는 말: 간흡충, cDNA library, ESTs, DNA 백신, 지방산 결합 단백질, 시스테인 단백 분해 효소, IgG2a, IFN-γ, Th-1.