Vaccine Research
ISSN: 2423-4923
eISSN: 2383-2819

Immunogenicity and Protective Capacity of *Schistosoma haematobium* Recombinant cathepsin L Against Infection of Hamsters with *S. haematobium*

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ARTICLE INFO

Original Article

VacRes, 2019
Vol. 6, No.2, 1- 8
Received: January 15, 2020
Accepted:May 16, 2020
Pasteur Institute of Iran

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KEYWORDS: *Schistosoma haematobium*, Hamsters, Cysteine peptidases, Vaccine, Recombinant proteins, Antibody response

ABSTRACT

Introduction: Vaccination of hamsters with *Schistosoma mansoni* adjuvant-free recombinant cathepsin B1 (SmCB1) and L3 (SmCL3) have been shown to elicit highly significant ($P < 0.005$) protection against challenge *Schistosoma haematobium* that was not very superior to that achieved by the cysteine peptidase, papain. Sterilizing immunity might, however, be induced if hamsters were vaccinated against S. haematobium infection with a homologous cysteine peptidase, i.e., S. haematobium cathepsin L (ShCL). **Methods:** Standards methods, techniques, and primers based on the published nucleotide sequence of ShCL were used to clone, amplify and express DNAs encoding the target enzyme in a bacterial expression vector. Repeat immunization trials were performed using recombinant ShCL alone or in combination with the vaccine candidate *S. mansoni* recombinant glyceraldehyde 3-phosphate dehydrogenase, in parallel with *S. mansoni* lecucine aminopeptidase. **Results:** The results together indicated that our adjuvant-free, cysteine peptidase-based vaccine elicits highly significant ($P < 0.0001$) reduction in challenge worm burden and parasite egg viability. Protection was associated with whole blood cultures release of type 1, type 2, and type 17 cytokines, and modest, yet significant ($P < 0.05$) humoral response to ShCL. **Conclusion:** Sterilizing immunity was, however, not achieved in any trial, likely because of the preponderant role of cysteine peptidases-induced nonspecific factors in amplifying and antagonizing its protective potential. Experiments are planned in an aim to identify these elusive factors and their exact role.

Citation:
Abdel Aziz N, Tallima H, Abou El Dahab M, El Ridi R. Immunogenicity and Protective Capacity of Schistosoma haematobium Recombinant cathepsin L Against Infection of Hamsters with S. haematobium. vacres. 2019; 6 (2) :1-8

INTRODUCTION

Schistosomiasis is a parasitic disease, endemic in 74 countries of the developing world with around 500 million people, mostly children, infected and 800 million at risk of the infection. Human infections are essentially due to *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*. Schistosomiasis haematobium is prevalent only in the Middle East and Africa; however, it is more prevalent (54.4%) than *schistosomiasis mansoni* (40.8%), especially in Western and Southern sub-Saharan African countries [1]. Infection is initiated by cercariae penetrating host epidermis where they remain until their classical trilaminate membrane transforms into a double lipid layer. The larvae, now termed schistosomula, access the dermis and then penetrate into the dermal blood capillaries en route to the liver via the pulmonary vasculature. The worms develop and mature in the liver sinusoids, copulate, and then leave to their permanent abode in the mesenteric capillaries (*S. mansoni, S. japonicum*) or vessels of the pelvic plexus (*S. haematobium*). Hundreds of eggs are deposited daily; about half of them exit via stools or urine to infect specific freshwater snails and continue the life cycle. The rest are trapped into the host tissues, releasing antigens which initiate vigorous inflammatory immune responses that are responsible for the disease symptoms [1- 3]. No commercial vaccine is available as yet.

The numerous molecules released, excreted and/or secreted (ESP) by the developing and migrating larvae are readily presented to the host lymphocytes, driving the induction of T helper (Th) effector cells and specific antibody-producing lymphocytes, and are accessible targets to the diverse immune effectors. Our Laboratory has identified many of the *S. mansoni* lung-stage larvae ESP, among which glyceraldehyde 3-phosphate dehydrogenase (SG3PDH), 14-3-3-like protein, 2-Cys peroxiredoxin (PRX = thioredoxin peroxidase, TPX), and calpain which are vaccine candidates are renowned for their immunogenicity and protective capacity [4]. We have proposed a mechanism of innate and acquired immunity-mediated schistosome attrition, whereby the larvae ESP are the "scent" which would allow the antibodies and immune cells to target, "hunt", pursue, and surround the migrating parasites. Interaction of antibodies and immune cells with ESP trigger the

[DOI: 10.29252/vacres.6.2.1]

generation of inflammatory mediators and toxic radicals that may eventually lead to elimination of the crawling parasites [4-6]. This "chase" is certainly most effective in the narrow blood capillaries of the lung and the sinusoids of the liver, long-known to be the major sites of natural and immune elimination of schistosomes [4-6].

Accordingly, it was important to revise the major issue of defining the global cytokine profile conducive to effective schistosome larvae killing. We proposed a paradigm shift in vaccination against murine schistosomiasis and delineated the steps necessary for development of a sterilizing vaccine against *schistosomiasis mansoni*. We have provided evidence for our hypothesis stating that the vaccine formula for an effective schistosomiasis vaccine would use larval ESP in the context of a polarized type 2, not type 1, cytokine environment. Indeed immunizing outbred, akin to man, mice with recombinant SG3PDH (rSG3PDH) and TPX-derived peptides in a multiple antigen peptide (MAP) construct, in combination with the type 2-inducing papain, or the type 2 cytokines thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, or IL-33 reproducibly and consistently elicited highly significant ($P < 0.0001$) 60-75% reduction in challenge worm burden and worm egg counts in liver and small intestine [7]. Our hypothesis was fully confirmed as immunization of outbred mice or hamsters with schistosome molecules, that are both ESP and type 2 immune responses-inducing, namely papain or the parasite cysteine peptidases, consistently and reproducibly elicited highly significant ($P < 0.0001$) reduction (of 50-60%) of challenge schistosome worm burden and worm egg load in liver and intestine as compared to unimmunized hosts [4, 8-13]. More specifically, hamsters immunized with *S. mansoni* cathepsin B1 (SmCB1) or L3 (SmCL3) displayed at day 10 post- challenge infection low antibody titers and weak but polarized cytokine type 2 responses to the immunogens. Both cysteine peptidases, alone or in combination, evoked reproducible and highly significant reduction in challenge worm burden (>70%, $P < 0.005$) as well as significant reduction in worm egg counts and viability [13].

It is herein aimed to increase the protective potential of the adjuvant-free, cysteine peptidase-based vaccine against *S. haematobium* via cloning the homologous cathepsin L, and examining its potential, alone or in combination with SG3PDH, in driving hamster immune responses towards the type 2 axis and ameliorating challenge *S. haematobium* infection sequelae.

MATERIALS AND METHODS

Cloning and Expression of *S. haematobium* Cathepsin L (ShCL)

Primer selection was based on published sequences for *S. haematobium* cathepsin L (gi|844841648; XM_012937844.1; XP_012793298.1 [14]. The mature product (648 bp) was targeted with forward primer [(5'-C ACC ATA CCA AAA AAC TTC GAT TG-3' (24 mer)], and reverse primer [(5'-CTA GTA GAT CAA CGC TGA C-3' (19 mer)], synthesized at Integrated DNA Technologies (IDT DNA, Leuven, Belgium) with additional CACC base pairs at the 5' of the forward primer, necessary for cloning in the selected expression vector (see below).

For cloning and amplification of cDNA coding for *S. haematobium* cathepsin L (ShCL) mature protein, RNA was extracted from 50 mixed-sex mature *S. haematobium* adult worms by using PureZOL (Bio-Rad, Hercules, CA, USA). Double stranded cDNA generation and amplification by RT-PCR were performed using SuperScript III One-Step RT-PCR System with Platinum® Taq High Fidelity kit (Thermo Fisher Scientific, Waltham, MA, USA), which produces blunt-end PCR products. Each sample (10 µl) was analyzed by 1.5% agarose gel electrophoresis and ultraviolet (UV) trans illumination (Biometra, Göttingen, Germany) using standard procedures, described previously [4]. As the amplicon was of the expected size, the cDNA was isolated from the agarose band by Quantum Prep Freeze N Squeeze DNA Gel Extraction Spin Column (Bio-Rad). Purity and concentration of the purified product were assessed by agarose gel electrophoresis and UV trans illumination. DNA sequencing of the amplicon was performed at the University of Cape Town Core DNA Sequencing Service (Cape Town, South Africa).

The purified ShCL-coding cDNA was expressed in the Champion pET Directional TOPO Expression Kit® (InVitrogen; Carlsbad, CA, USA), following the manufacturer's instructions which required blunt-end PCR products containing CACC at the 5' end, cloning into TOPO® vector, transformation and amplification in TOPO10 chemically competent *Escherichia coli*, preparation of plasmid, and then transformation and expression in the BL21 Star (DE3) chemically competent *E. coli*. The transformation reaction was grown in 200 mL LB medium containing 100 µg/ml ampicillin (Sigma-Aldrich, St. Louis, MO, USA) until the OD600 reached 0.7 (mid-log phase), and incubated for 4 h at 37˚C with shaking (300 rpm) in the presence of 1 mM IPTG. The induced cells were sedimented, incubated in Dulbecco's phosphate-buffered saline, pH 7.1 (D-PBS) supplemented with 1 µg/ml leupeptin and 1 mM PMSF as protease inhibitors, RNase T1 (100 µg/ml) and DNase 1 (20 µg/ml; all from Sigma-Aldrich) and sonicated (50 Watts for 2 min on ice). The suspension was then centrifuged at 10000 x g for 20 min and the soluble supernatant stored at -76˚C. The sedimented bacteria were incubated for 15 min at room temperature in D-PBS containing 25% sucrose and 1% Triton-X100 (Bio-Rad), sonicated at 50 Watts for 2 min on ice and then centrifuged as above. The Triton soluble supernatant was stored at -76˚C. Aliquots of uninduced and IPTG-induced bacteria and soluble and Triton soluble supernatants of cultures were analyzed by SDS-PAGE (10% gel, reducing conditions).

The recombinant protein was purified from the soluble fraction by metal affinity chromatography using HiTrap Chelating HP 5 ml columns (Amersham Biosciences AB, Uppsala, Sweden), following the manufacturer's instructions. The bound molecules were eluted and dialyzed overnight against 1x sterile D-PBS at 10°C. Protein content in the purified fraction was measured by the Bradford assay and by spectrometry whereby the protein was measured at absorbance 280 nm (A280) and A260 (UV-Photometer GeneRay, Biometra) and the protein concentration was calculated by the formula: protein concentration (mg/ml) = (1.55 x A280) – (0.76 x A260). Cathepsin L activity was determined by the fluorometric substrate assay as described [15-17]. Briefly, ShCL samples (10 and 50 µg) were incubated in duplicate wells of black 96 well plates (Greiner Bio-One, Kremsmünster, Austria), in parallel with 10 and 50 µg of SmCL3 (courtesy of Dr. Jan Dvořák and Professor Dr. John P Dalton) in a total volume of 250 µl of substrate/buffer mix (105.6 mM sodium mono phosphate, 14.4 mM disodium phosphate, 1.2 mM ethylenediamine tetraacetic (pH. 6.0), 2.5 mM L-cysteine, 0.07% (v/v) Brij 35, 0.006 mM Z-Phe-Arg-AMC(benzyloxycarbonyl-phenylalanylarginine- 7-amido-4-methylcoumarin hydrochloride; Sigma) or Z-Arg-Arg-AMC substrate at 40oC for 30 min. Release of free AMC was

[DOI: 10.29252/vacres.6.2.1]

measured at excitation and emission wavelengths of 355 and 460 nm, respectively, using Victor X4 Multilabel Plate Reader (PerkinElmer, Waltham, MA).

Ethics Statement

All animal experiments were performed following the recommendations of the current edition of the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, USA, and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Science, Cairo University, permit numbers CUFS F PHY 21 14 and CUFS-F-Imm-5-15.

Animals and Parasites

Female Syrian hamsters (Mesocricetus auratus) were raised at the Schistosome Biological Materials Supply Program, Theodore Bilharz Research Institute (SBSP/TBRI), Giza, Egypt, until 6 week-old, and were maintained throughout experimentation at the animal facility of the Zoology Department, Faculty of Science, Cairo University. Cercariae of an Egyptian strain of *S. haematobium* were obtained from SBSP/TBRI, and used immediately after shedding from Bulinus truncatus snails. Hamsters were anesthetized, the abdomen shaved, wet with sterile deionized water, and then exposed to 100 cercariae in 100 µl deionized water, protected from spilling by a sterile steel ring, as described [10, 12, 13].

Serum Antibody Assays

Blood samples were obtained from entirely naïve and from unimmunized and immunized hamsters (3 to 4 per group) 10 days (parasite lung-stage) following infection with *S. haematobium* cercariae. Sera were separated on an individual hamster basis, and stored at -20°C until use. Hamster serum antibody titer and isotype were assessed by indirect enzyme-linked immunosorbent assay (ELISA) for binding to 250 ng/well immunogen in duplicate wells as described [10, 12, 13]. Alkaline phosphatase (AKP)-labeled anti-hamster IgG (H+L) conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was diluted 1:1000. For each experiment, antibody isotypes of individual sera, diluted 1:50, was determined using biotin-labeled monoclonal antibodies to hamster IgG classes, IgG1 and IgG2 (Pharmingen, San Diego, CA, USA), and AKP-labeled streptavidin from Promega (Madison, WI, USA).

Whole Blood Cultures

Heparinized whole blood [18] 150 µl samples were diluted 1:4 in RPMI-1640 medium, supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin, 25 mM HEPES, and 20 µg/ml polymyxin B (Sigma-Aldrich) as an inhibitor of any lipopolysaccharide. Diluted blood samples were incubated in duplicate in wells of 48 well culture plates (Corning Costar, Corning, NY, USA) in the presence of 0 or 10 µg membrane (45 µm)-filtered ShCL for 72 h at 37˚C/3% CO2. Cultures were then centrifuged at 400 x g for 10 min and the cell-free supernatants transferred into wells of sterile plate, and stored at -76˚C until assayed for cytokine release.

Cytokine Assays

Serum and culture supernatants were assayed by capture ELISA in duplicate for levels of released hamster IL-4, IL-5, IL-13, IL-17, and IFN-γ (MyBioSource, Inc., San Diego, CA, USA) following the manufacturer's instructions.

Parasitological Parameters

Worm burden as well as liver and intestine worm egg load in individual hamsters (5 to 8 per group) were evaluated 12 weeks post infection with viable *S. haematobium* cercariae. Percent change was evaluated by the formula: % change = [mean number in infected controls − mean number in infected, treated hamsters / mean number in infected controls] × 100. Percentages of egg developmental stages were evaluated using 3-5 fragments of the ileum and the large intestine. After washing in saline solution and slight drying on absorbent paper, each intestinal fragment was placed between 2 slides and analyzed by light microscopy to classify the eggs. For each fragment, up to 100 eggs were counted and classified according to their developmental stage as immature, viable eggs; mature, viable eggs; and non-viable calcified eggs. Liver sections from each control and test hamster were stained with haematoxylin and eosin and examined for the number and diameter of granulomas surrounding eggs [12, 13].

Experimental Design

For Experiment 1, a total of 20 female Syrian hamsters were randomly distributed into two groups of 10 hamsters. Hamsters were subcutaneously injected at the tail base region, twice, with a 3 week-interval, with 200 µl D-PBS containing 0 (control group), or 20 µg *S. haematobium* cathepsin L (ShCL). Eight weeks after the second immunization, the control and immunized hamsters were percutaneously exposed to 100 cercariae of *S. haematobium*, and parasitological parameters were evaluated 12 weeks post challenge infection, as described [10, 12, 13].

For Experiment 2, 3 of 41 female Syrian hamsters were left unimmunized and uninfected and considered naïve animals. A total of 38 hamsters were randomly assigned to 3 groups of 10 hamsters each and a group of 8 hosts. Hamsters were subcutaneously injected at the tail base region, twice, with a 3-week-interval, with 200 µl D-PBS containing 0 (control group), 10 µg recombinant (r) ShCL, 5 µg ShCL plus 5 µg rSG3PDH (10 hamsters per group) and 8 hamsters with 10 µg rleucine aminopeptidase (a gift of Professor Dr. John Dalton). Five weeks after the second immunization, the control and immunized hamsters were percutaneously exposed to 100 cercariae of *S. haematobium*. On day 10, when a large proportion of migrating larvae are in the lung capillaries, serum and whole blood cultures supernatants from each of 2-3 hamsters/group were assessed for immunological responses to the immunogen. Parasitological parameters were evaluated for 5-8 hamsters per group, 12 weeks post challenge infection [10, 12, 13].

Statistical Analyses

All values were tested for normality. ANOVA, Mann–Whitney or Student's t 2-tailed test were used to analyze the statistical significance of differences between experimental and control values and considered significant at $P < 0.05$ (GraphPad InStat, San Diego, CA, USA).

RESULTS

ShCL Gene Cloning and Expression

The product obtained following gene cloning and amplification was analyzed by 1.5% agarose gel electrophoresis, and was of the expected size (648 bp) as shown before (Fig. 1A) and after (Fig. 1B) purification. DNA sequencing, translation, and blasting of the deduced amino acid

[DOI: 10.29252/vacres.6.2.1]

sequencing revealed 98% homology of the amplicon with *S. haematobium* cathepsin L at amino acid level (Fig. 2A, B). The induced product was found essentially in the soluble but also in the Triton soluble compartments around 30 kDa (Fig. 3). The recombinant protein was purified from the soluble fraction by metal affinity chromatography. The bound molecules were eluted and then dialyzed overnight against 1x sterile D-PBS. Purity and molecular mass of 2.0 µg of the purified product is shown in Fig. 4.

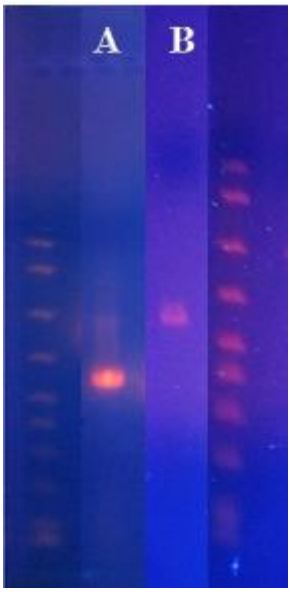


Fig. 1. Amplicon cDNA profile. Agarose gel electrophoresis was used to examine amplicon cDNA size and purity before (A) and after (B) purification, in parallel with DNA AmpliSize Molecular Ruler of Bio-Rad, from top 2000, 1500, 1000, 700, 500, 400, 300, 200, 100, 50 bp. The mature ShCL cDNA is seen between 500 and 700 bp marks (640 bp).

CGGCCKCTACTGAGTAAAAATCAAGGAATGTGTG
GCTCTTGTTGGGCGTTCTCAACCACTGGTAATGTT
GAGAGTCAGTGGTTCCGCAAAACTGGAAAGTTAT
TATCATTAAGTGAGCAGCAACTTGTTGACTGYGA
CGGTTTAGATGATGGTTGTAATGGTGGTCTTCCAT
CGAATGCCTATGAATCAATCATAAAAATGGGTGG
TTTAATGCTTGAAGATAATTATCCTTATGATGCTA
AAAATGAGAAATGTCATTTGAAAGCTGATRATGT
TGCAGCTTACATTAATAGTTCAGTAAATTTAACTC
AAGATGAAACAGAACTTGCTGCATGGCTTTATCA
TAATTCARCGATTAGTGTTGGTATGAATGCGWTG
CTTTTACAGTTTTATCGACATGGAATTAGTCATCC
ATGGTGGATCTTCTGTTCAAAGTATCTGCTTGATC
ATGCGGTTTTACTTGTTGGCTATGGAGTTTCCGAA
RAAAATGAGCCMTTCTGGATTGTAAAAAACAGTT
GGGGTGTTGAATGGGGTGAAAAAGGTTACTTCCG
TGTGTATCGAGGTGATGGTACTTGTGGCATTAATA
CTGTTGCTACGTCAGCGTTAWTYCTACTAGA

Fig. 2. (A). The Amplicon DNA sequence; 617 bp.

M C G S C W A F S T T G N V E S Q W F R K T G K L L S L S
E Q Q L V D X D G L D D G C N G G L P S N A Y E S I I K M
G G L M L E D N Y P Y D A K N E K C H L K A D X V A A Y
I N S S V N L T Q D E T E L A A W L Y H N S X I S V G M N
A X L L Q F Y R H G I S H P W W I F C S K Y L L D H A V L
L V G Y G V S E X N E X F W I V K N S W G V E W G E K G
Y F R V Y R G D G T C G I N T V A T S A L X L L

Fig. 2. (B) The amplicon cDNA translation. Using Expasy translate tool, the amplicon sequence yielded a polypeptide of 195 amino acids, which displayed 98% homology with *S. haematobium* cathepsin L (XP_012793298.1).

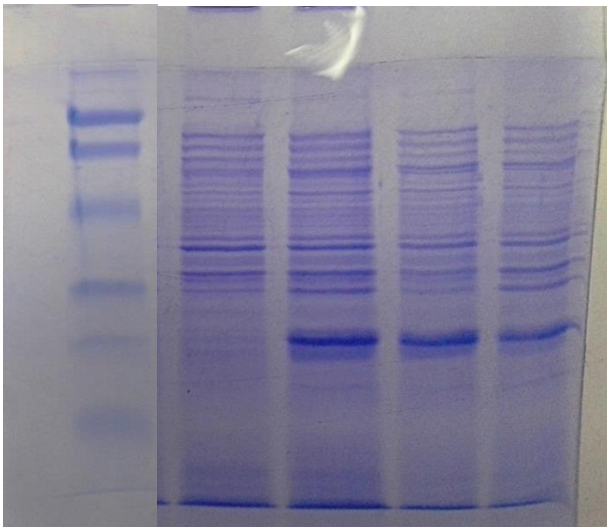


Fig. 3. Amplicon expression profile. Samples of uninduced (lane 1), IPTG-induced (lane 2), D-PBS-soluble (lane 3), and Triton-soluble (lane 4) bacterial molecules were separated by SDS-PAGE, and stained with Coomassie Blue. On left (lane M) prestained protein molecular mass markers of 20.486, 29.059, 36.811, 50.443, 84.174 and 105.203 kDa (Bio-Rad). The arrow points to the induced mature ShCL product, of around 30 kDa, present in D-PBS-soluble and Triton-soluble supernatants.

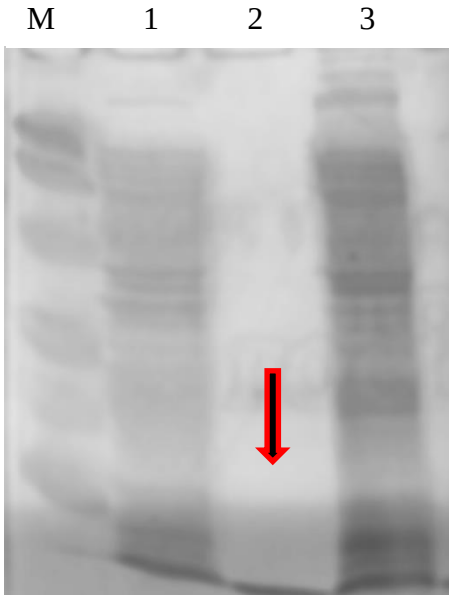


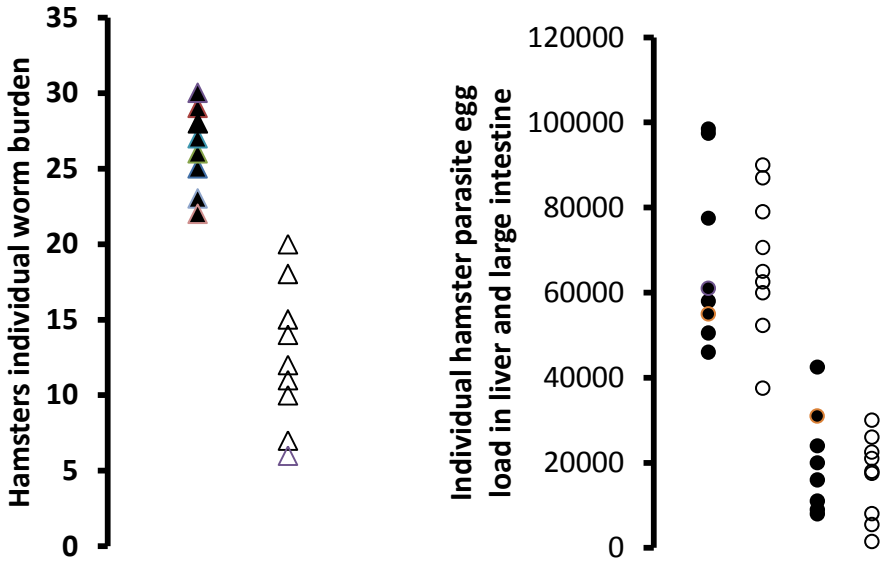
Fig. 4. Purified recombinant product profile. Samples of uninduced (lane 1), IPTG-induced (lane 3) soluble supernatants of recombinant bacteria, and the nickel affinity column-purified product molecules (lane 2) were separated by SDS-PAGE, and stained with Coomassie Blue. On left (lane M), prestained protein molecular mass markers of 20.486, 29.059, 36.811, 50.443, 84.174 and 105.203 kDa (Bio-Rad). The arrow points to the purified mature ShCL product, of around 30 kDa.

[DOI: 10.29252/vacres.6.2.1]

Of note, duplicate experiments indicated that ShCL displays approximately 10% of the activity shown by SmCL3 *in* fluorometric substrate assay for Cathepsin L activity [17].

Effects of Immunization with Recombinant ShCL on the Parasite Burden

Recombinant ShCL elicited highly significant ($P < 0.0001$) reduction of 52.3% in total worm burden (Fig. 5). Immunization with ShCL did not greatly reduce the number of eggs in the liver or large intestine (Fig. 5); yet, was associated with significant ($P < 0.02$) decrease in the percent of mature ova, and highly significant ($P = 0.0002$) increase in the percentage of dead ova that reached the large intestine (Table 1). Most importantly, immunization with ShCL resulted into significant ($P < 0.02$) decrease in the number and highly significant ($P < 0.0001$) reduction in the diameter of granulomas (Table 2), in support of the oogram information on the low viability of parasite eggs in the vaccinated group (Table 1).



			Liver		Large intestine	
Mean	26.2	12.5	68000	67094	20187	16666
SD	2.8	4.5	20700	16759	11982	9682
***P* value**		<0.0001		NS		NS

Fig. 5. Effect of immunization with recombinant mature ShCL on challenge worm and egg burden. Each point represents values in individual unimmunized (black fill) and ShCL-immunized (no fill) hamsters. Significance of differences between vaccinated versus infection controls was assessed by Student's-"t"- and Mann-Whitney tests. NS = not significant.

Table 1. Effect of immunization with ShCL on egg development of challenge S. haematobium infection in hamsters*.

Animal group			Mean % ova developmental stages ± SE			
	Immature	**P**	**Mature**	**P**	**Dead**	**P**
Controls	22.18 ± 8.20		65.72 ± 11.66		12.06 ± 4.05	
ShCL	21.68 ± 4.24	NS	31.38 ± 6.67	0.033	46.68 ± 3.26	0.0002

* Hamsters were vaccinated with ShCL, challenged 8 weeks after second immunization with 100 cercariae of S. haematobium, and assessed for parasitological parameters twelve weeks post infection whereby 10 fragments of large intestine were examined. A minimum of 300 eggs were counted for each animal. Significance of differences of percentages of different developmental stages in large intestine of vaccinated versus infection controls was assessed by Student's-"t"- test. NS = not significant.

Table 2. Effect of immunization with ShCL on liver granulomas induced by challenge *S. haematobium* in hamsters*.

Granulomas	Controls	ShCL
Number Mean ± SD *P* value Reduction %	83.1 ± 39.4	44.2 ± 19.3 0.018 46.8
Diameter Mean ± SD *P* value Reduction %	329.2 ± 52.1	169.4 ± 34.8 <0.0001 48.5

*Hamsters were vaccinated with ShCL, challenged 8 weeks after second immunization with 100 cercariae of S. haematobium, and assessed for number and diameter of granulomas in liver. The data show mean + SD of granuloma parameters in 10 fields (x100) of 3 slides for each control and vaccinated hamster. NS = not significant, as assessed by the Student's t- test (two-tailed P value). Reduction % = mean number in unimmunized hamsters – mean number in cysteine peptidase-immunized hamsters/ mean number in unimmunized hamsters x 100.

[DOI: 10.29252/vacres.6.2.1]

Cytokines Responses to Recombinant ShCL

Whole blood cultures from 3 naïve unimmunized and uninfected hamsters failed to produce detectable levels of IL-4, IL-5, IL-13, IL-17 or IFN-γ. The first and second order cytokine responses to *S. haematobium* were reflected, respectively in unstimulated and ShCL-stimulated whole blood cultures tested 10 days after infection, and involved release of significant amounts of IL-13 and IL-17. Whole blood cultures of ShCL-immunized hamsters expressed memory responses to the immunogen as judged by release of IL-13 and IFN-γ. The memory response to ShCL of ShCL + SG3PDH-immunized hamsters was evidenced by release of significantly ($P < 0.05$) higher levels of IL-4, IL-13, IL-17, and IFN-γ as compared to unstimulated cultures

Ten day infected hamsters did not produce detectable levels of anti-ShCL antibodies (data not shown).

Effects of Immunization with Recombinant ShCL alone or with SG3PDH

Immunization with ShCL alone or in conjunction with SG3PDH elicited increase in hamster serum antibody binding to ShCL (Fig. 6), involving essentially the IgG2 isotype. Regarding parasitological findings, ShCL alone or in combination with SG3PDH elicited highly significant ($P = 0.0007$ and $P = 0.0003$) reduction in total, male, and female worm burden, with highest decrease (65%; P (Mann-Whitney) = 0.0003) achieved in hamsters immunized with ShCL + SG3PDH. The highly significant ($P < 0.001$) reduction in *S. haematobium* worm numbers achieved following immunization with the cysteine peptidase-based vaccine is emphasized by the modest protective capacity ($P < 0.05$; 31%) of leucine amino peptidase (leucine AP) assayed in a parallel hamsters group (Table3).

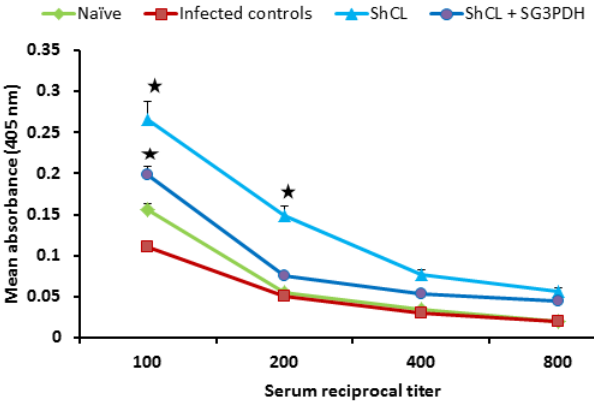


Fig. 6. Hamster serum antibody response to ShCL. Each point represents mean + SD absorbance of sera from 3 to 4 hamsters per group, assayed in quadruplicates by ELISA. Asterisks indicate significant ($P < 0.05$, ANOVA and "t" test)) differences from unimmunized infected controls.

Table 3. Effect of immunization with ShCL alone or in combination with rSG3PDH on parasitological parameters of challenge *S. haematobium* infection in hamsters*.

PARAMETER	VACCINE			
	Infected controls	ShCL	ShCL+SG3PDH	Leucine AP
Total worm burden Mean ± SD *P* value Reduction %	33.7 ± 9.0	13.5 ± 2.1 0.0007 60.0	11.7 ± 4.5 0.0003 65.2	23.2 ± 3.8 0.0325 31.1
Male worm burden Mean ± SD P value Reduction %	17.2 ± 5.0	6.6 ± 1.3 0.0007 61.6	5.5 ± 1.9 0.0003 68.0	11.6 ± 2.3 0.0397 32.5
Female worm burden Mean ± SD P value Reduction %	16.5 ± 4.2	6.8 ± 1.4 0.0007 58.7	6.1 ± 2.8 0.0003 63.0	11.6 ±1.9 0.0342 29.6
Liver egg counts Mean ± SD *P* value Reduction %	37875 ± 8412	27083 ± 6304 0.021 28.5	42357 ± 8385 NS	23500 ± 5024 0.0101 33.5
Large int. egg counts Mean ± SD *P* value Reduction %	8500 ± 6595	9750 ± 2824 NS	2857 ± 1435 0.048 66.3	2200 ± 1303 0.033 74.1

*Hamsters were vaccinated with ShCL alone or in combination with SG3PDH or with control antigen, leucine aminopeptidase (Leucine AP), challenged 5 weeks after second immunization with 100 cercariae of *S. haematobium*, and assessed for parasitological parameters twelve weeks post infection. Reduction % = mean number in unimmunized hamsters – mean number in immunized hamsters/mean number in unimmunized hamsters x 100. Data were statistically analyzed by ANOVA and the Mann-Whitney tests (two-tailed *P* value).

[DOI: 10.29252/vacres.6.2.1]

Table 4. Effect of immunization with ShCL alone or in combination with SG3PDH on development of challenge *S. haematobium* eggs in hamsters*.

Animal group			Mean % egg developmental stages ± SE			
	immature ova	P-value	Mature ova	P-value	Dead ova	P-value (Change%)
Control	53.49 ± 21.11		49.64 ± 19.35		9.04 ± 5.24	
ShCL	24.75 ± 24.09	NS	51.26 ± 23.36	NS	28.94±19.08	0.023 (68%)
ShCL + SG3PDH	27.90 ± 10.84	NS	15.67 ± 5.75	0.02*	60.85±12.87	0.0001 (85%)
Leucine AP	60.83 ± 13.16	NS	42.88 ± 31.08	NS	10.00 ± 5.23	NS

*Five weeks after vaccination, control and immunized hamsters were challenged with 100 cercariae of *S. haematobium*, and assessed for parasitological parameters twelve weeks post infection whereby 10 fragments of large intestine were examined. A minimum of 300 eggs were counted for each animal. Leucine AP = leucine aminopeptidase. Significance of differences of percentages of different developmental stages in large intestine of vaccinated versus infection controls was assessed by Student's "t"- test. NS = not significant.

The experimental data revealed that our cysteine peptidase-based vaccine does not elicit considerable decrease in challenge worm egg deposition (Table 3), but in sharp contrast to leucine amino peptidase effect, the great majority of the produced eggs are dead, especially if SG3PDH is included in the vaccine formula (Table 4). In support, significant ($P < 0.05$) decrease in egg counts was observed in large intestine of ShCL + SG3PDH-immunized hamsters (Table 3).

DISCUSSION

Despite that hamster immunization with *S. mansoni* cysteine peptidases elicited reduction in challenge *S. haematobium* burden that reached >70%, we thought that sterilizing immunity would be induced if we vaccinated hamsters with a homologous cysteine peptidase, i.e., *S. haematobium* cathepsin L (ShCL). Accordingly, standards methods and techniques and primers based on the published nucleotide sequence of ShCL allowed us to successfully clone and amplify cDNA encoding the target enzyme, which displayed 98% amino acid homology with *S. haematobium* cathepsin L. Expression in a bacterial vector resulted in production of a 30 kDa molecule that lacked distinct peptidase activity. None of 3 recombinant *S. mansoni* cathepsins B and L which had been previously expressed in *E. coli* exhibited detectable proteolytic activity. Absence of activity was expected as these enzymes and our recombinant protein possess many cysteine residues which have difficulty in refolding within the reducing milieu of the *E. coli* system in order to form 6 disulphide bonds [17, 19-21]. Expression in yeast is recommended for helminth derived cysteine peptidases [8-10, 17, 19-21]. Despite this lack of distinct proteolytic activity, ShCL induced a highly significant ($P < 0.0001$, Mann-Whitney test) reduction of 50-60% in *S. haematobium* challenge worm burden in hamsters, in the range of that of the enzymatically active SmCB1 and SmCL3 [13]. This result is in accord with our proposal that both active and inactive cysteine peptidases possess anti-schistosomiasis protective potential [22]. It is remarkable that SmCL3 (ABV71063.1) [17] consistently displayed protective potential against challenge *S. haematobium* infection in hamsters [13] despite showing only 39% identities and 56% positives with ShCL. *S. mansoni* cathepsin B1 (CAD44624.1) [21] was even more protective than SmCL3 [13] and ShCL1in that model, while showing with ShCL 26% identities and 41% positives. The data together support our suggestion that the cysteine peptidase vaccine protective activity is essentially due to shared conformational or linear motifs, epitopes, determinants in the cysteine peptidases polypeptides, in addition to, or independently of their enzymatic activity [22].

SmCB1, SmCL3 [13] or ShCL all failed to elicit decrease in liver egg load and highly significant reduction in large intestine egg count. Yet, the induced innate and/or acquired immunity led to considerable impairment of egg development and viability, resulting in the highly significant decrease ($P < 0.0001$) in size of liver granulomas. Of note, hamster immunization with *S. mansoni* leucine amino peptidase led to a modest decrease in challenge *S. haematobium* worm burden, and entirely failed to alter egg development and viability.

The mechanism of the cysteine peptidases anti-schistosomiasis protective mechanism appears to not depend on the activity of specific antibodies at the lung-stage, likely because memory responses would not be elicited before the time the worms grow, begin to eat and release the enzymes, at the liver stage [23]. Nevertheless, the immune responses results confirmed the poor immunogenicity of the cysteine peptidases of the L family and their ability to elicit type 1, type 2, and type 17 cytokines [8-10, 13, 17].

Addition of SG3PDH consistently led to increase in the protective potential of our cysteine peptidase-based vaccine [7-12]. The reason perhaps resides in the fact that this enzyme is released by schistosomes larvae immediately upon infection, as well as, developing, and adult worms, inducing memory responses at the lung- and liver stages [8, 11]. Attrition of worms at the lung- and liver stage [5, 6, 24] explains the higher reduction in worm burden and egg load in large intestine and the increase in percent of dead ova in intestine upon including SG3PDH in the vaccine formula. Immune responses elicited by ShCL alone or combined with SG3PDH were obviously limited, perhaps explaining why sterilizing immunity was not achieved. Additionally, it is possible that, contrary to leucine amino peptidase, our cysteine-based vaccine elicited predominant type 2 immune responses that resulted into generation of non-specific defense factors, which promote, yet also antagonize the cysteine peptidases-mediated schistosomicidal and ovocidal effects [22, 25].

ACKNOWLEDGEMENT

Thanks are due to Professor John Dalton (Queen University Belfast, Northern Ireland, UK) for the gift of *S. mansoni* recombinant leucine aminopeptidase and cathepsin L3. The

[DOI: 10.29252/vacres.6.2.1]

research was funded by the Science and Technology Development Fund (STDF) grant ID. 13874 to R. El Ridi and H. Tallima.

CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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[DOI: 10.29252/vacres.6.2.1]