Article

Identification and Characterization of Peptides Mimicking the Epitopes of Metalloprotease of *Schistosoma Japonicum*

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In an attempt to isolate and characterize peptides mimicking epitopes of metalloprotease and explore their immunological protection against *Schistosoma japonicum* (*S. japonicum*), polyclonal anti-metalloprotease sera was prepared to screen a 12-mer random peptide library to isolate phages binding specially to antisera IgG. Then, phage ELISA, animal immunization, DNA sequencing, Western blotting and enzymatic activity neutralizing analysis were used to characterize the selected phage clones. All of ten randomly picked clones were shown to be positive. Five peptides of different amino acid sequences deduced from DNA sequences were obtained and two of them (peptides 2 and 3) could induce significant reduction (31.0% and 31.8%, respectively) in worm burden and high reduction (52.6% and 54.9%, respectively) in liver eggs per gram (LEPG), while, unexpectedly, others (peptides 1, 4 and 5) could not elicit enough protection against infection of *S. japonicum*. Peptides 2 and 3 could be recognized by *S. japonicum* infected mouse sera (IMS) and could elicit neutralizing Abs. The results show that peptides 2 and 3 are antigenic and immunogenic. They are true mimics of epitopes of metalloprotease and useful as novel vaccine candidates against *S. japonicum. Cellular & Molecular Immunology*. 2005;2(3):219-223.

Key Words: phage displayed peptide library, epitope, *Schistosoma japonicum*, metalloprotease

Introduction

A wide rang of proteases from diverse organisms, which play a role at different stages of *Schistosoma* life-cycle in the vertebrate host, have been reported (1-6). Of these, metalloprotease is considered very important for invasion and immunoevasion of *Schistosoma* aside from known general catabolic function and protein processing (7, 8). These implicate the ability of metalloprotease to serve as not only pathological agent but also as serodiagnostic marker and vaccine target.

Library of random peptides expressed on the surface of filamentous M13 phage provides vast pools divers molecular structures from which peptides with affinity for molecules involved in biological interactions can be isolated. This technology has been used to map epitopes on proteins and to find peptide mimics for non-peptide-binding ligand (9). In this study, we used anti-metalloprotease sera to pan a 12-mer random peptide library to obtain information about the binding specificity of antisera IgG to epitopes on metalloprotease. Peptides that bind to IgG might be expected to mimic the epitopes of metalloprotease. It was anticipated that these peptides would provide information about the fine structure of epitopes of metalloprotease and might be proved useful vaccine candidates against schistosomiasis.

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Materials and Methods

Phage library and bacteria

The phage display library (a diversity of 1.5×10^{13} clones) and *Escherichia coli* (*E. coli*) host strain ER2738 were kindly provided by Dr. Larry McReynolds, New England Biolabs Inc.

Parasites and experimental animals

Cercarie of *Scistosoma japonicum* (*S. japonicum*) was obtained from *Oncomelania hupensis* which was purchased from Jiangsu Institute of Parasitic Disease, China. Female Kunming mice were provided by the Department of Experimental Animal, Xiangya School of Medicine, Central South University.

Antisera IgG preparation

The 85 kD metalloprotease of *S. japonicum* was fractioned in 10% discontinuous sodium dodecyl sulphate poly-acrylamidegel electrophoresis (SDS-PAGE) gel (6) and the gel was used to immunize rabbit for three times. Polyclonal rabbit antisera against metalloprotease was collected after the third immunization. Specific IgG was purified by saturated ammonium sulphate precipitation.

Panning the phage library

The panning technique (10) was adapted to screen the phage peptide library. Briefly, each microtiter well was first coated with 10 μg of IgG in 100 μl of coating buffer (0.1 mol/L $NaHCO_3$, pH 8.5) overnight at 4°C in sealed humid container, then blocked with 350 μl of 5% skimmed milk powder in TBS (50 mmol/L Tris, 150 mmol/L NaCl, pH 7.5) for 2 h at room temperature. Afterwards, 100 μl of original phages (containing 10^{12} phage particles) was added to the wells and incubated for 1 h at 37°C with gentle agitation. Following that, the wells were washed eight times with TBS-T (5 ml/L Tween-20 in TBS) to remove unbound phages. Phages that specifically bound to IgG were eluted with 100 μl of 0.2 mol/L glycine-HCl (pH 2.2) for 8 min at room temperature. Neutralized with 15 μl Tris-HCl (1 mol/L, pH 9.1), the eluted phages were titered and used to infect *E.coli* 2738 cells for amplification for the next round of panning. Three rounds of panning were performed.

Monoclonal phage amplification

Monoclonal phages randomly chosen from phage pools were amplified by infecting a log-phase culture of *E. coli* ER2738 and shaking for 4.5 h at 37°C in Luria-Bertani medium containing 0.02 g/L tetracycline. The supernatant was clarified by centrifuging at 10,000 rpm for 15 min and 1/6 volume of PEG solution (200 g/L polyethylene glycol 8000, 2.5 mol/L NaCl) was added. After incubated at 4°C for at least 2 h, the sample was centrifuged at 10,000 rpm for 15 min to precipitate the phage. The phage pellets were suspended in 1 ml TBS and stored at 4°C.

Phage ELISA

ELISA plates were coated with rabbit antisera IgG (10 μg/well) overnight at 4°C and then blocked with 5% skimmed milk powder for 2 h at room temperature. Phage particles (10^{12}) diluted in TBS were added to the wells and incubated at room temperature for 2 h. The wells were subsequently washed three times with TBS-T, followed by addition of anti-M13-HRP antibody. Specifically bound phages were visualized by the addition of 3,3,5,5-tetra-methylbenzidine (TMB). The absorbance was determined at 595 nm.

Immunoblotting

The 10^{12} phage particles from each selected clone were incubated in boiling water for 3 min in the sodium dodecyl sulfate (SDS) loading buffer and applied to 100 g/L SDS-polyacrylamide gels. Separated proteins were then transferred to nitrocellulose membrane and the membrane was blocked for 2 h at room temperature in 50 % skimmed milk powder in PBS. The membrane was rinsed three times with PBS and probed with mouse infection sera. HRP-linked anti-mouse IgG was used as a secondary antibody. Binding was visualized by incubating the membrane in diamino-bedzidine (DAB).

Table 1. Enrichment of phage clones during panning

Round of panning	Phage added (pfu/ml)	Phage eluted (pfu/ml)
1	2×10^{11}	2.6×10^{3}
2	2×10^{11}	4.5×10^{5}
3	2×10^{11}	1.8×10^{6}

Nucleotide sequencing

Single-stranded DNA was prepared from the purified phages as described by Sambrook et al (11). The nucleotide sequence of the gene III inserts was sequenced with -96gIII sequencing primer 5'-CCC TCA TAG TTA GCG TAA CG-3' by automated dye terminator cycle sequencing (ABI100). The aminoacid sequence of the insert was deduced from the nucleotide sequences.

Immunization and parasite challenge

Seventy 4-week-old female kunming mice were randomly divided into 7 groups. In the first 5 groups, mice were injected subcutaneously at three sites with 10^{12} individual phage clone particles respectively for three times. In control groups, mice were injected only with TBS or original library phage. Two weeks after the third immunization mice were challenged with 40 cercariae percutaneously on abdominal skin. All mice were perfused on day 42 postchallenge. The number of adult worms was determined and the liver eggs per gram (LEPG) were calculated.

Isolation of metalloprotease and Gelatin-substrate gel analysis

The method used by Yuan SS (6) was adopted to collect the excretory/secretory products (ES) of *S. japonicum* and to seperete the 85 kD metalloprotease. ES were fractionated in 10% discontinuous sodium dodecyl sulphate polyacrylamidegel electrophoresis (SDS-PAGE) gel slabs containing 0.1 % (w/v) gelatin under non-reducing conditions. Electrophoresis was performed at 4 °C, constant current 14 mA, in a Mini Protean System (Bio-Rad Ltd) with SDS-PAGE electrode buffer. Sample was mixed with non-reducing SDS-PAGE loading buffer and applied to the gel without boiling. After electrophoresis the gel were washed in 3× 100 ml changes of 2.5% (v/v) Triton X-100 for 30 min to remove the SDS and then sliced prior to incubation with buffers as following overnight: (A) 0.1 M Tris-HCl, pH 9.0; (B) 0.1 M Tris-HCl, pH 9.0, 1 mM EDTA. Three slices were incubated with immunosera (1:50 dilution) against peptides 2, 3 or M13 for 2 h at 37°C prior to with buffer (A). Proteolysis was visualized by Coomassie blue [0.1% (w/v) in 30:10:60 (v/v) methanol, acetic acid, water] staining followed by destaining in the same solvent.

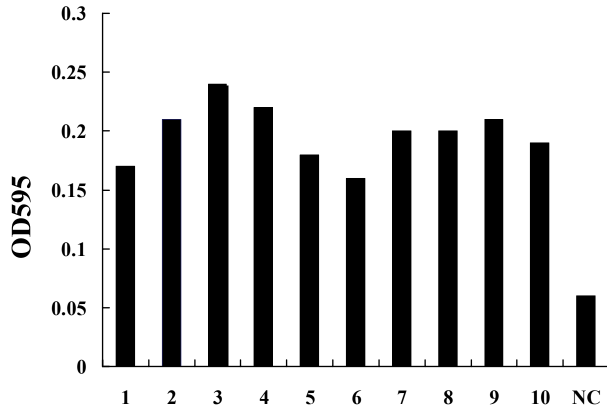

Figure 1. Identification of positive phage clones. The OD values of ten phage clones randomly selected were all twice more than that of negative control (NC) indicating that they were all positive clones.

Statistical analysis
Data analyses were performed with Statistical Package for the Social Science (SPSS, version 10.0). Data were shown as mean ± SD, and were considered statistically significant when $p < 0.05$.

Result

Enrichment of phage
Three rounds of panning resulted in a nearly 1,000 fold enrichment (Table 1). The phages ranged from 2.6×10^3 pfu/ml of the first elution to 1.8×10^6 pfu/ml of the third.

Identification of positive clones
Ten randomly selected individual clones were tested for their specific binding to IgG *via* phage ELISA. Original phage library was used as negative control (NC). When the OD value was twice more than that of NC, the phage clones were considered positive. The result showed that all of them had binding specificity to IgG (Figure 1).

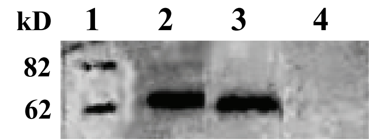

Figure 2. Analysis of interaction of phage clones 2 and 3 with *S. japonicum* infected mouse sera by western blotting. Tracks from left to right were: Lane 1, marker; Lane 2, phage clone 2; Lane 3, phage clone 3; Lane 4, M13.

Amino acid sequences of peptides insert in phages
Five DNA sequences were obtained and their amino acids were deduced (Table 2). The selected peptides did not share a common sequence motif.

Immunological protection against challenge infection with S. japonicum
Five phage clones of different peptide, severing as vaccines, were used to immunize female Kunming mice to assess their immune prophylactic potential against *S. japonicum*. We challenged the vaccinated mice two weeks after the third immunization and perfused them six weeks after challenge. The worms recovered and liver eggs were counted (Table 3).

Immunoblotting analysis
The peptides 2 and 3 were chosen and separated under nonreducing conditions by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes, the peptides fused gene III product were recognized by *S. japonicum* infected mouse sera (IMS) while wild-type M13, which did not display any foreign peptide, was not (Figure 2).

Gelatin substrate gel analysis
The 85 kD metalloprotease, which could hydrolyze gelatin

Table 2. Five DNA sequences and amino acid deduced

Peptides	DNA and amino acid sequences											
1	ACG T	TCG S	TTT F	GGT G	AGT S	ATG Met	CTT L	AGT S	AAG K	TGG W	CAG Q	AAG K
2	AGT S	AAT N	CCT P	CCG P	GGG G	ATG Met	GCT A	CTT L	TCG S	GCT A	CCG P	CCT P
3	ATT I	ACG T	TCG S	CAT H	ACG T	GGG G	TAT Y	CTG L	CAG Q	CTT L	CGT R	TTG L
4	ACT T	CTT L	GCT A	CAT H	ACT T	AGT S	CAG Q	ATT I	GGG G	CTT L	ACG T	GCT A
5	ATG Met	GAG E	GCT A	TCT S	CAT H	ACG T	CAT H	GCG A	CGT R	CCG P	GCG A	CCT P

Table 3. Reduction of worm burden and LEPG in mice vaccinated with each peptide

Groups	No. of worms recovered	Reduction *vs* TBS (%)	*p* value	LEPG (× 10^2)	Reduction *vs* TBS (%)	*p* value
1	23.6 ± 1.6733	10.6	0.055	99.36 ± 7.28	18.5	0.009
2	18.2 ± 1.9235	31	0.001	57.75 ± 3.62	52.6	0.001
3	18.0 ± 1.4142	31.8	0.001	54.94 ± 3.64	54.9	0.001
4	25.2 ± 1.7889	4.5	0.48	106.30 ± 3.84	12.8	0.145
5	24.2 ± 2.2804	8.3	0.073	102.40 ± 2.35	16	0.055
Original Phage	24.4 ± 1.8166	7.5	0.318	108.80 ± 3.46	10.7	0.2
TBS	26.4 ± 1.9494	-	-	121.86 ± 3.47	-	-

and be inhibited by EDTA, was fractioned (Figure 3). Another two hydrolysts about 47 and 55 kD were visualized, but they could not be inhibited by EDTA suggesting they are not metalloprotease.

If the peptide sequences mimic the epitopes of metalloprotease, the immunosera they derived would neutralize the metalloprotease activity. The efficacy at neutralizing the enzymatic activity to hydrolyze gelatin was shown in Figure 3. After neutralized by immunosera metalloprotease could not hydrolyze the gelatin while anti-M13 sera could not affect the activity of metalloprotease (Figure 3).

Discussion

Although much is known about metalloprotease in terms of its function, there is a paucity of information regarding its immunological property. In this report, we used metalloprotease immunosera to pan a 12-mer random peptide phage display library to identify peptides mimicking the epitopes of metalloprotease. After three rounds of panning, the phage pools were enriched nearly 1,000 fold. All of the ten randomly picked phage clones were shown to bind specifically to antisera IgG indicating that these phage clones would be able to mimic the reacting properties of epitopes with IgG.

None of the peptides identified in this study had any homology with others of *S. japonicum* in GenBank. It is not clear why there was no selection of peptides that closely resembled the sequences of *S. japonicum*. It is possible that what these peptides mimicked is the spatial structure of epitopes. Isolation of peptides that are not identical to the original target from random peptide library has been reported by others (12). Although the peptides were diverse in sequence, there was a significant number of Ala (11.7%) and Ser (13.3%) residues found in the five motifs while the expected frequencies in the naïve library are 6.2% and 9.4%, respectively. It would appear, therefore, that these residues play a critical role in interaction between antisera IgG and epitopes.

In order to test the mimics for their protective effect as vaccines, a challenge experiment was performed. In groups 2 (phage clone 2) and 3 (phage clone 3), there was a significant reduction in worm burden and even higher in LEPG (Table 2) suggesting that the two phage clones selected were peptides mainly directing at the output of eggs of *S. japonicum*. The similar result that phage peptide-based vaccine against *S. japonicum* was mainly directing the egg production has been reported by Ouyang L, et al. (13). Day TA, et al. (14) showed the egg production of adult worm pairs was inhibited by metalloprotease inhibitor 1,10-phenanthroline *in vitro*, we, in this article, showed the anti-metalloprotease mimics antibodies to reduce the egg laying *in vivo*. Unexpectedly, however, the others did not induce enough protective effects on worm burden. One possibility is that the titer of neutralizing antibodies elicited is not high enough (data not shown). What is more important is that maybe peptides 1, 4 and 5 are not the mimics of metalloprotease epitopes.

Peptides 2 and 3 were selected as antigens to test their ability to interaction specifically with IMS. The position of peptide fused to gpIII was visualized indicating that the two peptides were antigenic (Figure 2).

To confirm that the peptides (2 and 3) were true mimics of structural features of the epitopes, the capacity of sera to inhibit metalloprotease activity was analysed (Figure 3). The

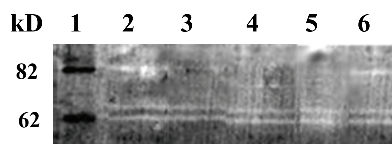

Figure 3. Gelatin substrate gel showing the function of the Abs. Metalloprotease (85 kD) was fractioned in gelatin SDS-PAGE under nonreducing condition from excretory/secretory products (ES) and the capacity of sera to nutralize metalloprotease activity was analysed. Lane 1, protein mark; Lane 2, control, incubated in buffer (0.1 M Tris-HCl, pH 8.8); Lane 3, slice incubated with peptide 2 immunoserum prior to incubation with buffer; Lane 4, slice incubated with peptide 3 immunoserum prior to incubation with buffer; Lane 5, metalloprotease inhibitor, 1 mM in buffer; Lane 6, slice incubated with M13 immunoserum prior to incubation with buffer.

result showed that after neutralized by sera, metalloprotease could not hydrolyze gelatin, demonstrating that the peptides shared the properties of metalloprotease epitopes. The use of gelatin substrate analysis as model allowed us to investigate the quality of the Ab response in term of enzymatic neutralizing capacity.

In summary, our results demonstrate that phage peptides 2 and 3 can mimic features of epitopes of metalloprotease and they are antigenic and immunogenic and proved useful candidates of vaccines against schistosomiasis.

Acknowledgements

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