Proteome approach for identification of schistosomiasis japonica vaccine candidate antigen

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Abbreviation: RAC, radiated-attenuated cercariae; NCI, normal cercariae infected; NC, healthy control; SEA, soluble egg antigen; SWAP, schistosomal worm antigen preparation; HPCF, high performance chromatofocusing; HPRP, high resolution reversed phase chromatography.

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Abstract

Experimental vaccination with radiation-attenuated cercariae (RAC) confers possible practical levels of resistance to challenge infection by humoral and by cellular mechanism. Here, we aimed to identify possible vaccine antigens by using specific IgG antibody from RAC vaccinated miniature pig. Two milligrams of soluble egg antigen (SEA) or schistosomal worm antigen preparation (SWAP) was fractionated using two dimensional liquid chromatography (proteome PF 2D) consisted of high performance chromatofocusing (HPCF) and high resolution reversed phase chromatography (HPRP). Of the 42 HPCF fractions of SEA or SWAP, 26 (61.9%) or 15 (35.7%) showed positive dot blot reaction with RAC vaccinated serum respectively. The dot blot positive fractions were applied to the second HPRP column. One hundred and seven out of 26 x 96 of SEA fractions and 18 out of 15 x 96 SWAP fractions reacted with RAC vaccinated serum. From the positive fractions we chose 17 of SEA and 10 of SWAP that had no reactivity with normal cercariae infected (NCI) sera and had single peak of 214 nm; and automated N-terminal amino acid sequence based on in situ Edman Reaction was conducted. Four sequences were obtained and applied to the homology search in NCBI database. A total of eight candidate genes were listed up and their cDNA clones from schistosomula stage were obtained. Two of the recombinant proteins (AAW27472.1 and AXX25883.1) showed strong reactivity with the RAC vaccinated serum but marginal with NCI serum. This protocol using proteome PF2D could be applicable in identifying immunoreactive proteins from crude extract for the development of vaccines or for diagnostics.

Keywords: *Schistosoma japonicum*; Radiation-attenuated cercariae; vaccine; miniature pig; proteome

1. Introduction

Schistosomiasis is a parasitic disease which affects more than 200 million individuals in Africa, Asia and South America. It is endemic in 74 countries causing more than 250,000 deaths per year [1]. Despite two decades of comprehensive campaign for the control, the number of individuals with active schistosomiasis worldwide remains at about 200 million annually [2,3]. Vaccine has long been expected to be developed as a novel strategic tool for the control [4─6]. A comprehensive review on current status of vaccines for schistosomaiasis had provided by McManus and Loukas [7].

There are three different approaches for isolation and identification of schistosome vaccine candidates [8,9]. Selection based on protective monoclonal antibodies such as glutathione-S-transferase (GST) [10] and triosephosphate isomerase (TPI) [11], by unique antigen recognition by strong natural resistance in humans [12] or animals [13], or by antigen selection using the radiation-attenuated cercariae vaccine (RAC) model [14─16]. For example, a fragment of myosin of *S. mansoni* (SmIrV-5); one of the vaccine candidate antigens selected by TDR/WHO committee [17], was identified using serum from mice exposed to RAC [18]. None of the antigens identified conferred equivalent efficacy of the vaccination using RAC [5,19,20]. There is a quantitative and/or qualitative difference between the immune responses generated by RAC vaccine and those by defined subunit vaccine [21,22]. Increased immunogenicity of RAC is related to delayed and truncated pattern of migration, in contrast to the normal parasite [23]. In fact, radiation induces defects in the neuromuscular coordination of the developing larvae [24]. Accordingly, delayed parasite migration through skin or skin draining lymph nodes and lungs would mean that there is a greater opportunity for interaction of parasite antigen with the immune cells at these sites which may in turn favor the priming of the protective response.

The highest levels of resistance obtained in mice after vaccination with RAC require antibodies and T cells [25,26]. It is clear that protective immunity generated by RAC vaccines is mediated by acquired immune mechanisms that require the reactivation of antigen specific CD4$^+$ helper cells [27]. However, passive transfer experiments indicate that IgG antibodies are also the key mediators of this immunity [28─30] .Thus, elucidation of antigens relevant to both humoral and cellular response may be critical for the development of an optimal vaccine.

Most of our knowledge on schistosomiasis is drawn from experiments in primates and rodents. Although primate model is relatively better but the high cost and ethical concerns make them difficult to be used [31]. Also the use of rodents has several problems as a model for schistosomiasis [32,33]. When we focus on RAC vaccine of *S. japonicum*, higher protection has been achieved in rhesus monkeys [34], in cattle [35,36] and in pigs [19]. Within the reservoir host animals, pig would be the easiest one compared with cattle or sheep, but still the major drawback of the domestic pig is the large body mass. For this reason we established a unique miniature pig model for human Schistosomiasis japonica [37]. In this study we aimed to identify the major antigenic molecules which are specifically recognized by RAC vaccinated serum of miniature pigs for identification of possible candidate schistosomiasis vaccine.

2. Material and methods

2.1. Parasite

Chinese strain of *S. japonicum* was obtained from Jiangsu Provincial Institute of Parasitic Diseases Wuxi, Jiangsu Province, People's Republic of China. Cercariae were released from the infected snails by light induction as described [38]. Attenuation of the cercariae was carried out by

200 Gy of γ-irradiation at a rate of 33 Gy / min using ^{60}Co irradiator (Pony Industry CO. LTD. PS-3100sB, Osaka, Japan).

2.2. Preparation of cultured schistosomula

Cercariae were released from 100 infected snails in a beaker containing 200 ml of tap water under light for 2 to 3 hours at room temperature and were passed through Sartorus Nylon spacer, mesh size 0.1 mm. The obtained suspension was centrifuged for 60 sec at 1500 rpm (383g) at 10 ^{o}C to concentrate cercariae. The cercariae were washed twice in Basal Medium Eagle (BME) (Gibco-Invitrogen Co., CA, USA) containing 15 mM of HEPES (Gibco-Invitrogen Co.), 300 units/l Penicillin (Gibco-Invitrogen Co.) , 300 µg/l Streptomycin (Gibco-Invitrogen Co.) and 160 µg/l Gentamicin (Gibco-Invitrogen Co.) in aseptic condition. Transformation of cercaria to schistosomula was carried out mechanically by shearing the tail using vortex for 3 min; and about 200-300 parasites/ml of the mechanically transformed shistosomula were cultured in DMEM (Sigma-Aldrich Co., MO, USA) containing 1% Fetal calf serum (FCS) (Hyclone, UT, USA), 300 units /l penicillin and 300 µg/l streptomycin in 24 well plate (Corning, NY, USA) at 37 ^{o}C in 5% CO_2 incubator for 24 h. The cultured schistosomulae were collected by centrifugation, washed three times with phosphate buffered saline (PBS) and kept frozen at - 80 ^{o}C until use [39].

2.3. Soluble worm antigen preparation (SWAP)

SWAP was obtained by the method described elsewhere [40]. Briefly, *S. japonicum* adult worms were obtained by perfusion of infected rabbits. After lyophilization, worms were homogenized in cold diethyl ether and centrifuged to remove lipids. The pellet was then freeze-thawed several times in PBS, pH 7.4 containing 1mM phenyl methyl-sulphonyl fluoride (PMSF)

and 2μg/ml Leupeptin (Sigma). The homogenate was dialyzed against several changes of PBS at 4°C, and centrifuged at 30,000g for 50 min at 4°C. The supernatant was filtrated through 0.22μm filter (Millipore Co., MA, USA) and this was used as SWAP. Protein concentration was determined by BCA protein Assay Kit (Pierce Biotechnology, Inc., IL, USA).

2.4. Soluble Egg antigen preparation (SEA)

Preparation of SEA was previously described [41]. Briefly, *S. japonicum* eggs were isolated from infected liver and intestine of rabbit. The purified eggs were finally adjusted to a concentration of 50,000 eggs/ml of PBS with 1mM of PMSF, and 2μg/ml Leupeptin (Sigma) and sonicated three times on ice for 10 min. The suspension was freeze-thawed several times and centrifuged at 30,000g for 50 min at 4°C. The supernatant was filtrated through 0.22μm filter; and this was used as SEA. Protein concentration was determined by BCA protein Assay.

2.5. Experimental animals, parasitological examinations and serum sample collection

Six-week-old male CLAWN strain miniature pigs (Japan farm, Kagoshima, Japan) weighing between 2.5 kg and 3 kg were used in this study. The pigs were fed with standard feed based on their body weights, with water ad libitum. Seven pigs were used, three for immunization with radiation-attenuated cercariae (RAC) of *S. japonicum*, three for infection with 200 cercariae (NCI) and one as a healthy control (NC). Sera were collected four weeks after vaccination and four weeks after challenge by drawing blood from auricular vein at indicated times. Before taking blood, pigs were anesthetized by intramuscular injection with 0.2 mg/kg midazolam (Yamanouchi Pharmaceutical Co., Ltd. Tokyo, Japan) and 40 μg/kg medetomidine (Orion Corp., Espoo, Finland). The first group of pigs was subjected to a single percutaneous exposure of 400 RAC using a cover slip as described [37]. Four weeks later, the first and second groups of pigs were

challenged with 200 normal cercariae (NCI). The third group was used as a healthy control. Feces were collected every week and the number of eggs excreted into feces was counted as previously described [37]. Adult worms were recovered from the liver and mesenteric veins as described [20]. The experimental protocol was approved by the Animal Ethical Committee of Nagasaki University (No.0204250127-3).

2.6. SDS-PAGE and Western blot analysis

SWAP and SEA were boiled for 5 min in reducing sample buffer containing 4% sodium dodecyl sulfate (SDS) and 50 mM Dithiothreitol (DTT), and electrophoresed by using 5-20% gradient SDS polyacrylamide gel (E-pagel®, Atto Co., Tokyo, Japan) [42]. For the western blot analysis, SEA and SWAP proteins separated by the E-pagel were transferred onto PVDF membrane (Millipore Co.). After blocking in 5% skimmed milk in Tris-buffer (TBS-Tween 20) (20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.1% Tween 20) for 1 h at room temperature, the protein transferred-membrane was incubated with the 3000 times diluted test serum for 2 h at room temperature. After washing three times with TBS-Tween 20, the membrane was incubated in 10 ml of a secondary antibody solution containing 40,000 times diluted affinity Purified anti-porcine IgG antibody that is horseradish peroxidase (HRP) conjugated (Anti pig IgG (H+L)-HRP, AP166P, Chemicon International, CA, USA) and TBS-Tween 20 for 1 h at room temperature. After washing three times with TBS-Tween 20, reactive protein bands were visualized by exposing an x-ray film using ECL-Plus Western blotting Detection system (G. E. Healthcare, Amersham Biosciences, Buckinghamshire, UK).

2.7. Protein purification and fractionation

The protein purification and fractionation of SWAP and SEA were performed using Proteome Lab PF 2D system (Beckman Coulter, CA, USA) that is designed for two dimensional liquid chromatography consisted of a high-performance chromatofocusing (HPCF) in the first dimension followed by high-resolution reversed-phase chromatography (HPRP) in the second dimension. One ml of 2 mg/ml of either SWAP or SEA was introduced with a manual injector into the column for the first dimensional HPCF. The protein was bound to a strong anion exchanger followed by elution with a continuously decreasing pH (8.5─4.0) gradient (Beckman Coulter). The proteins were eluted based on their isoelectric point (pI), collected in a 96 deep-well plate (Beckman Coulter) [43]. The first dimensional fractions were directly applied to the second HPRP in a C18 column. The mobile phase consisted of two buffers, the first one 0.1% Trifluoroacetic acid (TFA) in water and the second one 0.08% TFA in acetonitrile. Separation was performed according to the Manufacturer's instruction protocol.

2.8. Dot Blot ELISA

The fractions were blotted onto a PVDF membrane (Millipore Co.) using the Bio-Dot [®] SF Micro filtration apparatus (Bio Rad Laboratories, Inc., CA, USA). Briefly, 20 μl of each fraction were blotted onto the PVDF membrane and the membrane was soaked in 5% skimmed milk in Tris-buffer (TBS-Tween 20) for 1 h at room temperature. The membrane was incubated with 1000 times diluted test serum for 2 h at room temperature. The reactivity of each dot with test serum was estimated by the dot blot ELISA using the same method described above in the western blotting. We selected positive dots when they showed increased intensity compared with those incubated with normal control serum. The criteria that used for selection of the positive/negative results of dot-ELISA are using two positive controls, the crude antigens of SWAP and SEA, and

PBS as a negative control. All the positive fractions of the first dimension were sequentially applied to the second dimensional HPRP.

2.9. Amino acid sequencing

Fractions from the second dimensional HPRP were analyzed by dot blot ELISA and positive fraction was subjected to amino acid sequencing. Two hundred microliters of positive fraction was spotted onto Polybrene-coated glass fiber discs. Subsequently proteins were sequenced with an automated protein sequencer (ABI Model cLc; Applied Biosystem, CA, USA).

2.10. Homology search

The obtained amino-terminal sequences were blast-searched for their homology with the genes deposited in the *S. japonicum* database in NCBI using BLAST/ blastp suite programs, in non redundant Gene Bank Coding Sequence (CDS). After homologous genes were listed up, further selection was performed according to the following criteria; the homologous area should be located at the N-terminus sequences and its deduced pH must be within the pI range of first dimensional fraction.

2.11. mRNA analysis

To confirm mRNA expression of the candidate genes in the different stages of the parasites, total RNA was extracted from cercariae, 24 h cultured schistosomulae, eggs, and adult worms of *S. japonicum* according to the instruction manual of Micro-to-midi total RNA purification system kit (Invitrogen Co.). The first strand cDNA was synthesized from the total RNA by using oligo (dT) primer according to the instruction manual of high capacity cDNA reverse transcription Kit (Applied Bio systems) and was used as template for reverse transcript PCR using a set of primers

that were designed from the candidate gene as indicated in the footnote for Table 3. *S. japonicum* actin gene primers were used as an internal reference. RT-PCR was performed by the following condition of 42 cycles of 30 sec at 94 ºC, 50 sec at 55 ºC and 2 min at 72 ºC for all samples. PCR products were subsequently separated on 1% agarose gel, stained with ethidium bromide and visualized under UV light. The resulting PCR products were cloned into pCR 2.1 using TOPO-TA cloning Kit (Invitrogen Co.), sequenced using Big-Dye V.1.1 terminator cycle sequencing Kit (Applied Biosystem) and analyzed on an ABI 3710 DNA Sequencer (Applied Biosystem) for confirmation with database sequences.

2.12. Production of recombinant protein

Reverse-PCR products from cercarial mRNA purified from agarose gel were cloned into the pET/100 D-TOPO expression vector (Invitrogen Co.) and transformed into chemically competent TOP10 *Escherichia coli* (Invitrogen Co.) according to the manufacturer's protocol. Cells were plated onto LB-Ampicillin (50 μg/ml) plates and incubated for 18 h at 37 ºC. Ten positive clones were identified and were grown for 18 h in 6 ml LB medium containing Ampicillin (50 μg / ml) and plasmids prepared using a Gene Elut^{Tm} plasmid Miniprep kit (Sigma). Plasmids were then digested with Nhe I and Sac I (New England Biolabs, MA,USA) for 2 h at 37 °C and the inserts detected by separating the DNA on a 1% agarose gel stained with ethidium bromide and visualized under U.V. light.

To confirm the correct orientation and in frame, the inserts were sequenced using Big-Dye V.1.1 terminator cycle sequencing Kit (Applied Biosystem) and analyzed on an ABI 3710 DNA Sequencer (Applied Biosystem). Plasmid DNA containing expression constructs was transformed into BL21 Star™ (DE3) One Shot® *E. coli* (Invitrogen Co.) for recombinant protein expression. Briefly, 10 ng of plasmid DNA was transformed into the bacteria by heat shocking at 42 °C for 30

sec. Transformed bacteria were grown overnight at 37°C in LB medium supplemented with either 100 μg/ml ampicillin or 50 μg/ml carbenicillin prior to pilot expression by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer according to the manufacturer's protocol (Invitrogen Co.). Briefly, 10 ml of LB medium containing 100 μg/ml ampicillin or 50 μg/ml carbenicillin was inoculated with 500 μl of an overnight culture and allowed to grow for 2 h at 37°C with shaking until they reached mid-log phase of growth. IPTG was then added to a final concentration of 0.1mM to 0.5 mM and a 500 μl aliquot was removed from the culture, centrifuged at 10,000 g in a micro centrifuge for 30 sec. The supernatants were removed and the cell pellets frozen at - 20°C. Remaining cultures were incubated at 37 ℃ with shaking and 500 μl aliquots were removed after 1, 2, 4 and 6 h post-induction. Individual sample was analyzed by SDS-PAGE and Western blot for detection of recombinant protein using Anti–His G-HRP Antibody (Invitrogen Co.).

3. Results

3.1. Protective response in miniature pig vaccinated with Radiation-attenuated cercariae (RAC)

The mean of fecal egg excretion of RAC vaccinated group was significantly reduced at 6th, 8th and 9th weeks post infection, when compared to the control group as shown in Table 1. The recovered worm number after the perfusion also indicated the vaccination effect of RAC as shown in Table 2.

3.2. Antigen recognition by sera from the vaccinated miniature pigs

We aimed to identify the antigenic molecules reactive with RAC vaccinated serum of miniature pigs but not with serum from those just infected with 200 cercariae for 4 weeks. As a target antigen preparation, we used SEA and SWAP antigens [16].

Pooled sera obtained 4 weeks after the RAC vaccination recognized antigenic molecules of 263, 255, 155, 130, 78, 55 and 20 kDa from SEA as shown in lane (R) of Figure 1a. Whereas sera obtained 4 weeks post challenge with 200 normal cercariae, recognized antigens of 253, 155, 130, 74, 60, and 37.5 kDa (lane N of Figure 1a). When we used SWAP, pooled sera obtained 4 weeks after the RAC vaccination recognized antigens of 70, 27, 21 and 10 kDa as shown in lane R of Figure 1b. Sera obtained 4 weeks post challenge with normal cercariae recognized antigens of 150, 71, 36, 27, 21 and 10 kDa (lane N, Figure 1b). The pooled sera that used in this experiment were diluted 3000 times.

3.3. First dimension: high-performance chromatofocusing (HPCF)

Following the HPCF fractionation of SEA and SWAP 42 fractions were obtained. All the 42 fractions from SEA or SWAP were tested for reactivity with RAC vaccinated pooled serum as shown in Figure 2a and 2b. Totally 26 SEA and 15 SWAP fractions were reacted to RAC vaccinated pooled serum as shown in Table 3.

3.4. Second-dimension: high performance reversed-phase chromatography (HPRC)

Those 26 and 15 dot blot positive fractions of SEA and SWAP respectively, from the first dimension (HPCF) were subjected to further fractionation by the HPRC. The Proteome maps of the second dimensional separation are shown in Figure 3a and 3b.

Elution pattern of the fraction number 2 of SEA after the second column is shown in Figure 4a, as an example. All the fractions obtained after the second column were tested for

reactivity against the RAC vaccinated serum. Out of the 2,496 SEA and 1,440 SWAP fractions, 107 and 18 fractions reacted with the RAC vaccinated serum respectively, as shown in Table 3. These 107 and 18 positive fractions were further tested for reactivity with normal cercariae infected (NCI) serum. Out of the 107 SEA and18 SWAP fractions, 46 and 8 fractions reacted with NCI serum respectively (Table 3). Dot blot pattern for fraction number 2 of SEA after second column separation was shown in Figure 4b as an example. D3 and G5 fractions showed strong reactivity and D3 fraction had single retention peak while G5 fraction had two peaks as shown in Figure 4a. The pooled sera used in dot blotting experiments were diluted 1000 times.

3.5. *Amino acid sequencing*

As shown in Table 3, after identification of 107 SEA and 18 SWAP fractions, we selected 61 SEA and 10 SWAP fractions by excluding the fractions reactive to NCI serum. For amino acid sequencing, we analyzed their retention peak patterns as shown in Figure 4a, and picked 17 from SEA and 10 from SWAP that had single peak. Finally we obtained four N-terminal sequences, 2 from SEA and 2 from SWAP as shown in Table 4.

3.6. *Homology Search*

The four identified N-terminal amino acid sequences were applied to the NCBI /BLAST/ blastp suite programs. After the homology search we set the selection criteria of the candidate genes as follows, (1) high homology on the N- terminal sequences (2) deduced pH compatible with the pI range of first dimension. Two candidate genes for each amino acid sequence were selected as shown in Table 4.

3.7. *Expression of mRNA encoding the candidate genes in different developmental stages*

mRNA from eggs, cercariae, 24 h culture schistosomulae and adult worms of the Chinese strain of *S. japonicum* was used for RT-PCR amplification of the selected candidate genes. All the candidate genes were expressed in all the stages of the parasite, as shown in Figure 5, except for the genes AAW27155 that was expressed only in the egg stage and AAW26143.1 that is expressed in the egg, cercariae and adult but not in schistosomulae (Figure 5). All the amplified PCR fragments had exactly the expected molecular weight as in the NCBI database.

3.8. *Recombinant and native protein reactivity with RAC vaccinated serum*

Protein expression in *E coli* using recombinant pET100 / D-TOPO expression vector bearing each PCR fragment was confirmed by the detection of a band in Coomassie Brilliant blue stained SDS-PAGE after IPTG induction and by western blotting probed with Anti His-tag Antibody or with vaccinated sera (Data not shown). Two candidate genes named AAW27472.1 (Figure 6) and AAX25883.1 (Figure 7) with recombinant proteins having molecular weights 15.5 kDa and 24 kDa respectively were strongly recognized by RAC vaccinated sera but not reactive with NCI or NC sera. While the recombinant protein AAW27690.1whose molecular weight was 35 kDa showed weak reactivity with RAC vaccinated serum but strong reactivity with NCI as shown in Figure 7. Original native protein fractions of AAW27472.1, AAX25883.1 and AAW27690.1 that were F2.3D for AAW27472.1 and F12.1F for the latter two genes were analyzed by the western blotting analysis to compare with the recombinant proteins patterns. As is shown in Figure 6, native protein that corresponds to the same molecular weight around 11 kDa of the recombinant was detected by RAC serum.

Figure 7 also shows strong reactive band of around 18 KDa in the F12.1F native fraction with RAC serum but not with NCI serum that may correspond to the AAX25883.1 recombinant

protein of 21 kDa. Interestingly F12.1F fraction contained reactive band of 25 kDa with NCI serum that almost correspond to AAW27690.1 recombinant pattern.

4. Discussion

We have already reported that high levels of protective immunity were obtained by UV-attenuated cercaria vaccination in Chinese domestic pigs [20]. In this study we used Clawn miniature pigs for vaccination with RAC and observed a comparable level of protective immunity as seen in the domestic pigs (Table1). Previous studies have shown that serum of RAC vaccinated mice can transfer protective immunity to naive recipients [25,44]. We concentrated on the analysis of antibodies in the development of resistance in this RAC vaccine miniature pig model.
In the present study, sera from RAC vaccinated pigs reacted with a number of proteins of SEA whereas sera collected from NCI showed different pattern of reaction (Figure 1a).

The strong reactivity to SEA by the RAC vaccinated and NCI pigs could be explained by cross-reactivity of the provoked antibodies against components of cercaria to schistosomula stage worms or by the existence of common molecules shared between young worm and eggs. The significant difference of the reactive SEA proteins against RAC vaccinated and NCI sera suggests that the mode of immunization is different between those two methods [45,46].

It is well known that much of the antibodies of infected subjects are directed against carbohydrate determinants from the adult worms and the eggs [47]. We confirmed our major RAC vaccinated antibodies were directed against peptide by treating crude SEA and SWAP with glycopeptidase A, to remove the glycoconjugates. This treatment did not abolish the basic reactivity detected by the western blotting patterns of SEA or SWAP (data not shown).

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Then we tried to identify a series of unique proteins that are recognized by RAC vaccinated sera but not by normal cercaria infected sera (NCI). As shown in Figure 2 and Table 3, two-dimensional (2D) liquid chromatography successfully resolved the peptides that were shown to be reactive to RAC vaccinated serum. Although it is not clear that the 2D-LC has significant advantage over the 2D-SDS PAGE, using 2nd dimensional column, we could physically separate totally 2496 SEA fractions from 42 positive fractions of the first dimensional column and identified 107 fractions that were specifically reactive with RAC serum. Separation of intact proteins coupled with fraction collection was another advantage for this method [43]. Although our Edman degradation method could not identify many peptides, the discrimination ability from such a crude extract can be tolerated for further study.

We successfully obtained enough length of N-terminal sequences; 4 fractions out of 27 (Table 4). As shown in Figures 6 and 7, even after the 2D column separation, each fraction contained several proteins as visualized by silver staining or by western blotting. The overall efficiency of the N-terminal sequencing of the fractions by the Edman Reaction is dependant on the purity and quantity. So that, we might have picked up major peptide's sequences in a terms of quantity. Of course there is a fact that many schistosomal proteins were glycosylated and were possibly N-terminally blocked for the Edman Reaction. There is no doubt that MS-based protein identification is much faster and technically easier and currently the first choice over Edman degradation. Despite the fact that most of biological samples are a mixture even after physical separation as used in this study, the power of MS-based protein identification currently available should enable estimation of likeliness and relative abundance of identified proteins based on the number of peptides and coverage. The MS-based method must be more promising for this kind of identification.

After we got four sequences, the current database of ESTs from *S. japonicum* was used for the homology search. Unexpectedly, we have got only a limited level of homology to all the input sequences as was also noticed in the previous study [48]. Because of a large size of *S. japonicum* genome, it has not been subjected to full scale genome sequencing;[49,50]. Of course ESTs do not cover full length of the coding regions and a limited portion of transcripts are likely deposited in the EST database [51]. In order to increase the probability of selection of the candidate genes that were picked by their homology, pI was set as a second criterion for the selection (Table 4). His-tag fused recombinant proteins from the entire listed candidate genes were confirmed by western blotting using anti-his tag antibody and by their expected molecular weights, and only three recombinant proteins of AAW27472.1, AAX25883.1 and AAW27690.1 showed reactivity to RAC vaccinated serum.

The recombinant proteins obtained from the cloned AAW 27472.1 and AX25883.1 were highly reactive to RAC vaccinated serum but marginal with NCI serum that exactly reproduced the original fraction's behavior (Figure 6 and Figure 7). While the recombinant protein obtained from the cloned AAW27690.1 showed weak reactivity with RAC vaccinated serum, it showed strong reactivity with NCI as shown in Figure 7. Therefore, AX25883.1 is more probable to match with the obtained N-terminal sequence.

The expected molecular weight of AAW27472.1 was 12.475kDa but the protein in original purified fraction showed about 11 kDa as shown in Figure 6. This may be a result of post-translation modification that often occurs in schistosomes [52]. AAW27472.1 was revealed to be a hypothetical protein, but had 23% homology with Cathepsin B endopeptidase *(S. japonicum)*, and had 26% homology with cathepsin B endopeptidase (*S. mansoni*). In contrast to AAW27472.1, the expected molecular weight of AAX25883.1 was 18 kDa, and the protein in original purified fraction showed same molecular weight as shown in Figure 7. AAX25883.1 was revealed to be a

Syntaxin N-terminus domain that is a neuron system-specific protein implicated in the docking of synaptic vesicles with the presynaptic plasma membrane and had 23% homology with crystal structure of the 26 kDa glutathione S-transeferase of S. *japonicum*. The expected molecular weight of AAW27690.1 was 25 kDa, and the protein in original purified fraction, showed the same molecular weight as expected as shown in Figure 7. AAW27690.1 was revealed to be a NADH ubiquinone oxidoreductase subunit of NDUFA12 and had 26% homology with "NADH dehydrogenase subunit 5". As the original purified fraction showed a reactive band around 25 kDa, this may be explained by the same reason as AAW27472.1.

In the present study, we have focused on the identification of soluble proteins and did not analyse the membrane protein that is expected to be another source of vaccine candidates. This 2D column system is applicable to the detergent solubilized membrane proteins using 2 % Trion X-100 [53]. Membrane protein must be the next target for our study.

Although we have not yet examined the efficacies of these subunit candidate vaccines, we concluded that our 2D column protein fractionation system was simple and effective to identify the immuno-reactive proteins from crude extract.

Acknowledgements

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Figure Legends

SEA
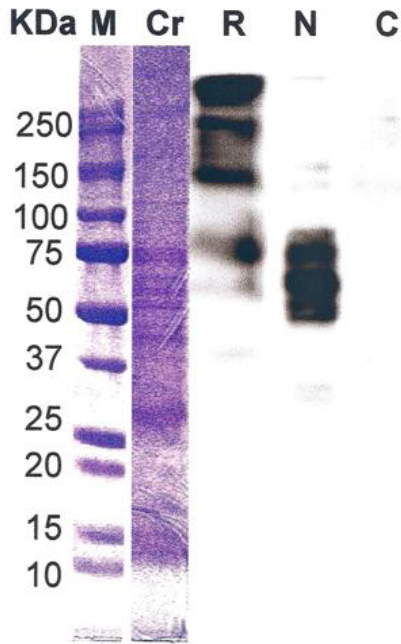
SWAP
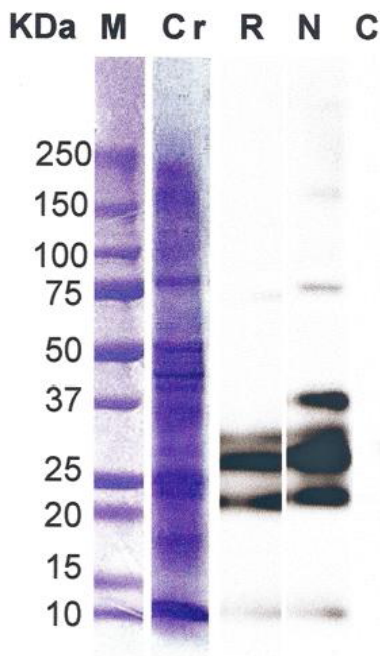
Figure 1. SDS-PAGE and western blot analysis of RAC (R), NCI (N) and NC (C) sera of miniature pig for the specific IgG against (a) SEA and (b) SWAP.

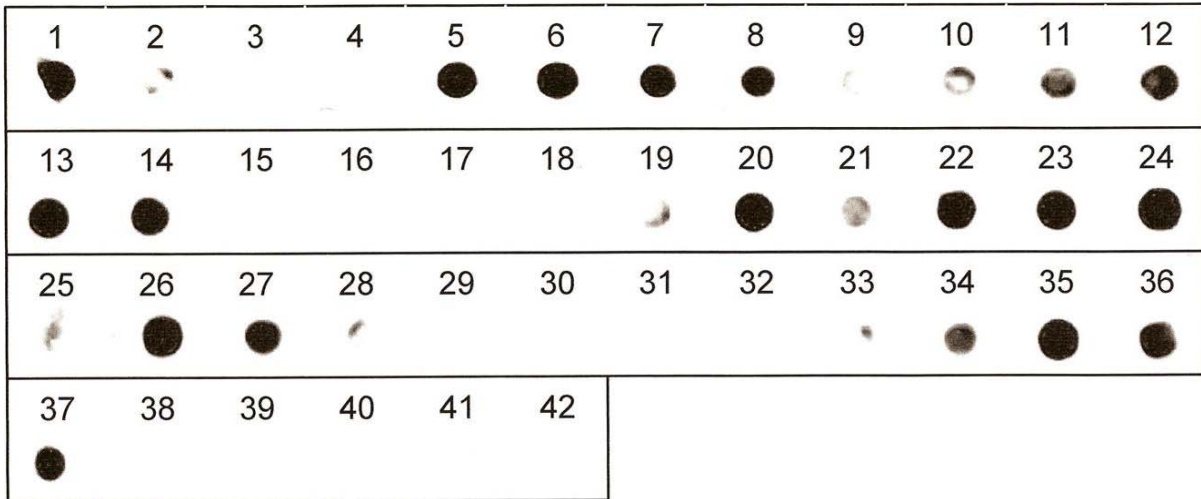Lane M: molecular weight marker; lane Cr: CBB staining of the crude extract; lanes R, N and C: Western blot pattern probed with a panel of sera; lane R: RAC serum; lane: N
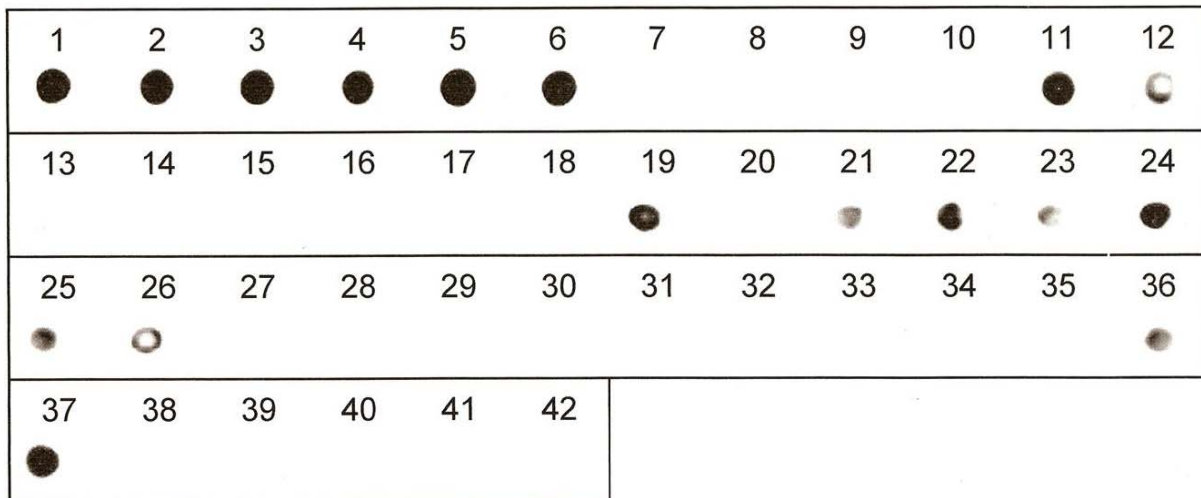
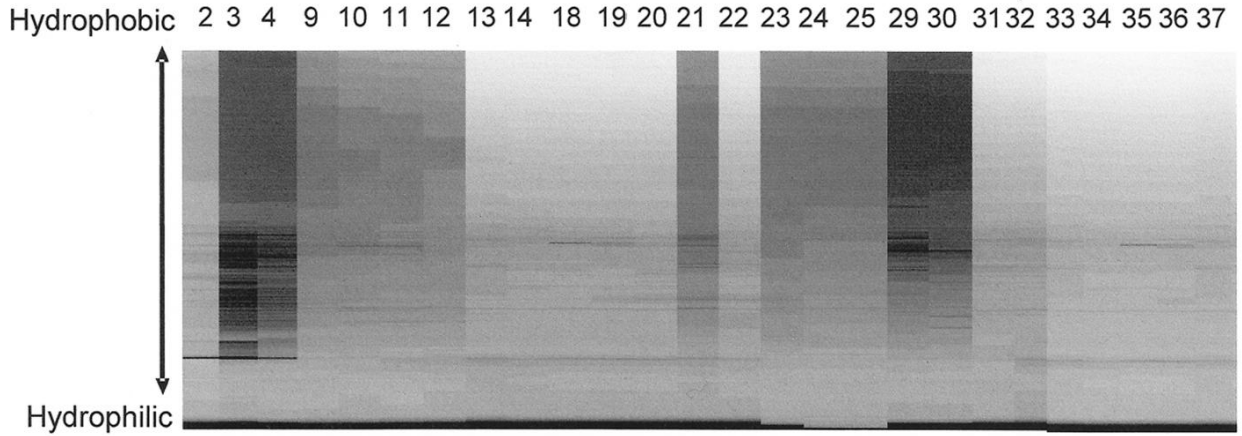NCI serum and lane: C NC serum. Sera were diluted 3000 times.
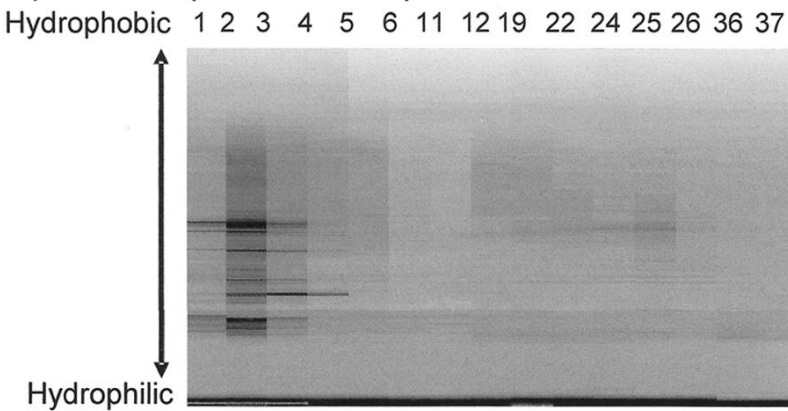
a) SEA

b) SWAP

Figure 2. Dot blot analysis of (a) SEA and (b) SWAP fractions of HPCF using pooled RAC vaccinated sera were diluted 1000 times.

Dot blot has been repeated twice and the representable positive fractions to RAC serum were selected.

a) SEA (26 fractions)

Hydrophobic   2   3   4   9   10   11   12   13   14   18   19   20   21   22   23   24   25   29   30   31   32   33   34   35   36   37

Hydrophilic

b) SWAP (15 fractions)

Hydrophobic   1   2   3   4   5   6   11   12   19   22   24   25   26   36   37

Hydrophilic

Figure 3. The Proteo Vue ® MAP of the second dimensional separation for the positive dot blot fractions from SEA (a) and SWAP (b).

Hydrophobicity of each fraction is expressed by its retention time. Each band represents Optical Density of a peptide at 214 nm. The fraction numbers of the first column are shown on the top of the figures.
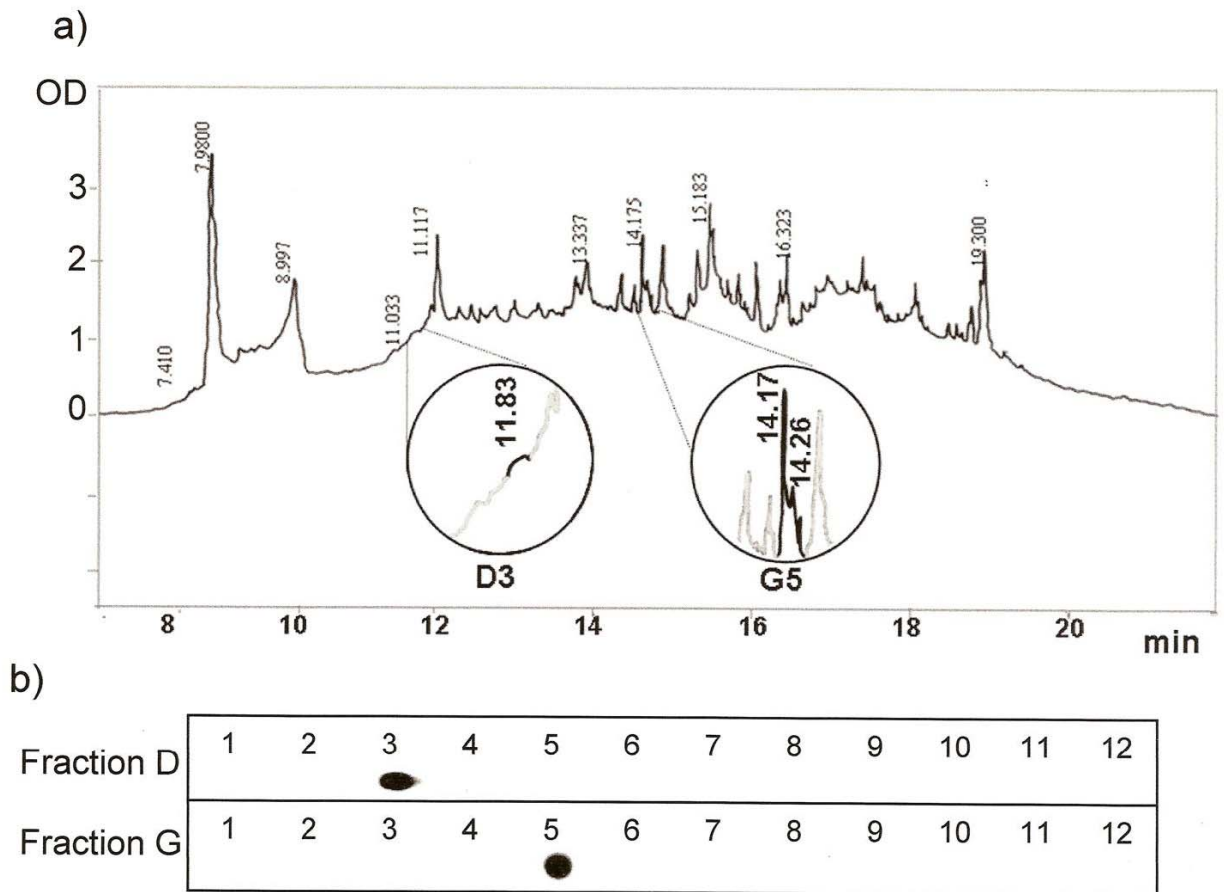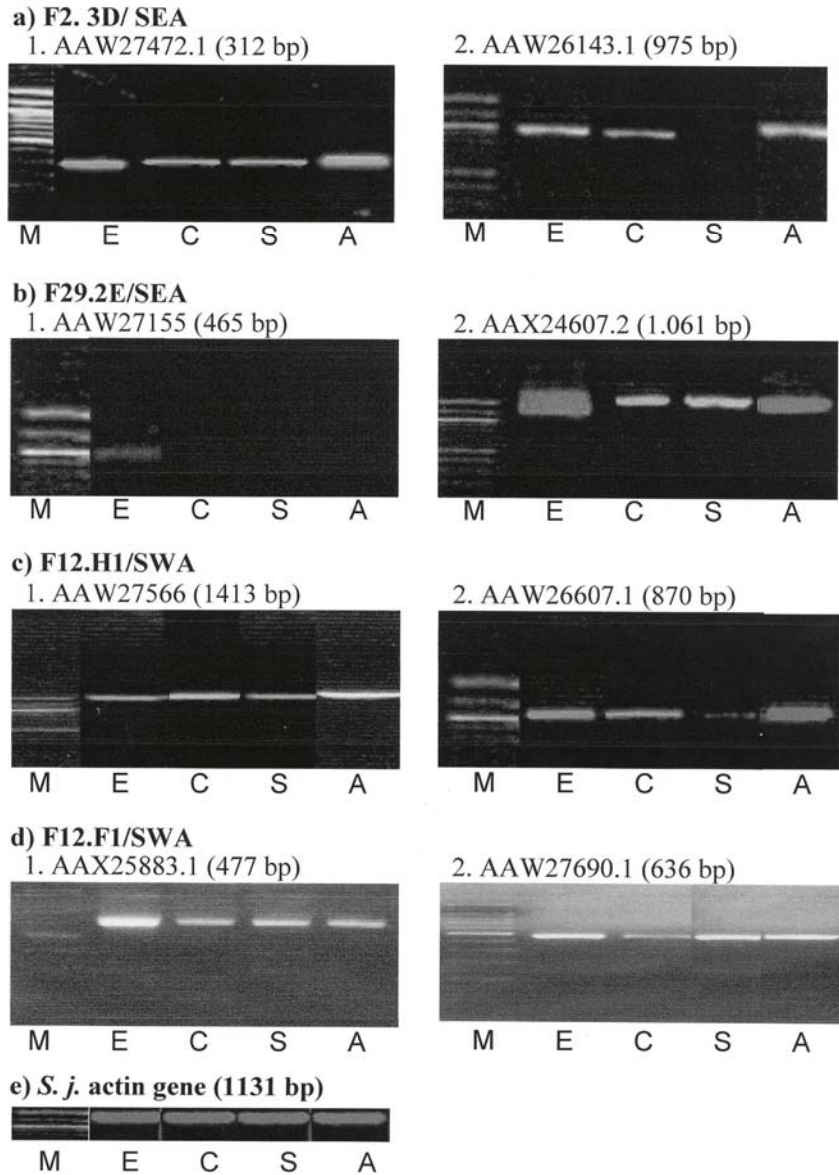
Figure 4. Representative of the second column separation of the first fractions (fraction No.2 of

SEA)(a) and the dot blots analysis for the fraction No. 2 of SEA(b).

(a) The optical densitometry pattern (OD at 214 nm) is represented. The x axis represents

retention

time in min according to the concentration of acetonitrile in the mobile phase.

(b) D3 and G5 are the two fractions obtained from the 96-well plate that was used for collection

of

the second column separation.

a) F2. 3D/ SEA
   1. AAW27472.1 (312 bp)   2. AAW26143.1 (975 bp)

b) F29.2E/SEA
   1. AAW27155 (465 bp)   2. AAX24607.2 (1.061 bp)

c) F12.H1/SWA
   1. AAW27566 (1413 bp)   2. AAW26607.1 (870 bp)

d) F12.F1/SWA
   1. AAX25883.1 (477 bp)   2. AAW27690.1 (636 bp)

e) *S. j.* actin gene (1131 bp)

Figure 5. mRNA expression of the selected candidate genes from different developmental stages of *S. japonicum.*

PCR fragments with expected size amplified from the different stages of the *S. japonicum* were obtained using primer sets designed for the eight candidate genes and the actin gene as a control. Lane M: molecular weight maker; lane E: egg; lane C: cercariae; lane S: 24hs cultured schistosomula; lane A: adult worm.

(e) *Schistosoma japonicum* actin gene.
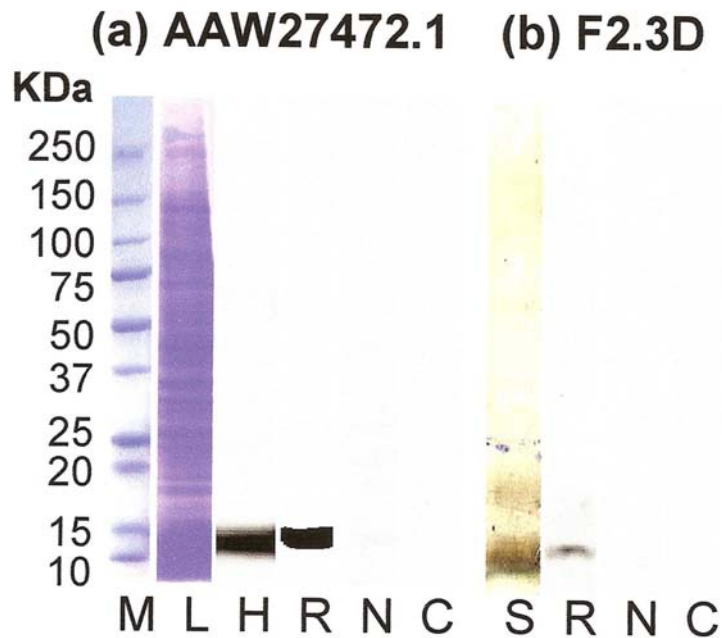
Figure 6. Reactivity of the recombinant protein of AAW27472.1 (a) and its original fraction,

F2.3D / SEA (b) with RAC vaccinated serum.

(a) Lane M: molecular weight marker; lane L: CBB stained pattern of total lysate after induction

with IPTG. Lanes H, R, N and C: Western blotting patterns of the total lysate probed with a panel

of sera; lane H: Anti histidine tag antibody; lane R: RAC serum; lane N: NCI serum; lane C: NC

serum.

(b) Lane S: Silver staining patterns of the original fraction, F2. 3D fraction. Lanes R, N and C:

Western blotting patterns of the F2. 3D fraction probed with a panel of sera; lane R: RAC serum;

lane N: NCI serum and lane C: NC serum.
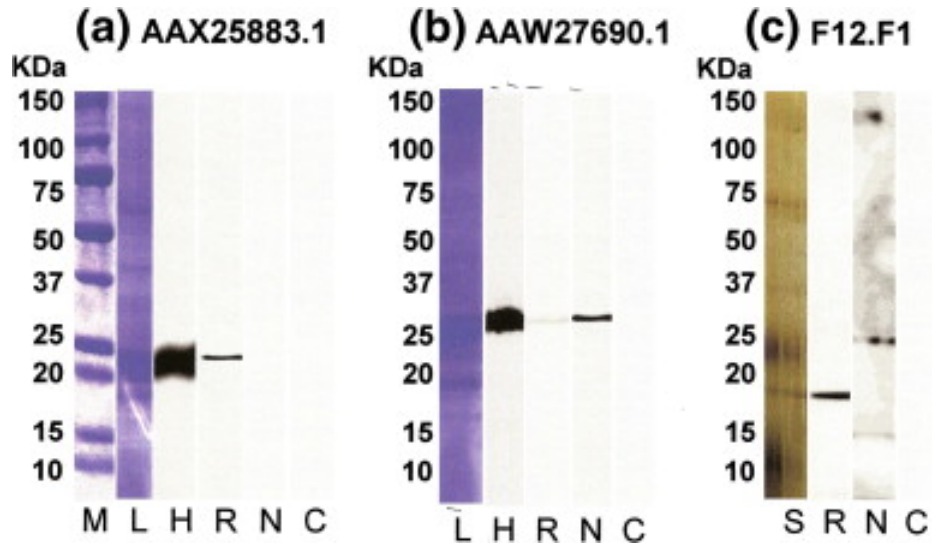
Figure 7. Reactivity with RAC vaccinated serum of the recombinant proteins of AAX25883.1(a) and AAW27690.1 (b) and that original fraction, F12.1F SWAP (c).

(a) and (b) Lane M: molecular weight marker; lanes L: CBB stained pattern of total lysate after induction with IPTG. Lanes H, R, N and C: Western blot patterns of the total lysate probed with a panel of sera; lanes H: Anti histidine tag antibody lanes R: RAC serum; lanes N: NCI serum and Lanes C: NC serum.

(c) Lane S: Silver staining patterns of the original fraction F12. F1 fraction). Lanes R, N and C: Western blotting patterns of the F12. F1 fraction probed with a panel of sera; lane R: RAC serum; lane N: NCI serum and lane C: NC serum.

Table 1: Fecal egg excretion of RAC immunized and none immunized miniature pigs after challenge infection with 200 cercariae of *S. japonicum* Chinese strain.

Examination time after challenge infection	EPG (egg per gram of feces)		P^c
	RA^a (n = 3)	NC^b (n = 3)	
5th week	74.34 ± 82.26	102.81 ± 81.47	NS
(EPG of individual)	(45.03, 196.00, 67.40)	(190.65, 18.65, 13.71)	
6th week	102.81 ± 81.47	413.42 ± 171.92	0.028
(EPG of individual)	(438.78, 193.48, 162.82)]	(610.00, 291.13, 339.14)	
7th week	180.68 ± 104.29	389.85 ± 254.99	NS
(EPG of individual)	(298.61, 142.86, 100.57)	(681.03, 206.42, 282.10)	
8th week	156.24 ± 99.28	558.26 ± 181.23	0.026
(EPG of individual)	(265.91, 130.36 ,72.46)	(686.22, 637.68, 350.88)	
9th week	207.42 ± 79.60	480.61 ± 76.57	0.003
(EPG of individual)	(280.49, 219.18, 122.95)	(528.69, 520.83, 392.31)	

[a] RA: vaccinated with 400 RAC.

[b] NC: no vaccination control.

[c] P values were calculated by two tailed Student's t -test.

The values in parentheses represent the EPG for each pig.

Table 2: Worm numbers recovered from each group of miniature pigs.

Pig group	Worm burden			
	Total	Male	Female	Reduction rate [b]
RA 1	5	4	1	96.4 %
RA 2	42	36	6	69.7 %
RA 3	51	44	7	63.2 %
Average	32.67	28.0	4.67	76.4 %
NC 1	160	135	25	—
NC 2	117	87	30	—
NC 3[a]	—	—	—	—
Average	138.5	111.0	27.5	—

NC3[a]: this pig has been died at the 9th week after infection due to sudden death.

Reduction rate [b]: The worm reduction rate = (the average worm burden in the control group─the worm burden in the vaccinated group)/(the average worm burden in the control group) x 100.

Table 3: Dot blot analysis of the first and second fractions of SEA and SWAP that reacted with RAC vaccinated or / and NCI serum from miniature pigs.

sequential screening steps		No. of the RAC positive fractions (%)	
		SEA (%)	SWAP (%)
1st dimension	(42 Frs. of SEA, 42 Frs. of SWA)	26 (61.9)	15 (35.7)
2nd dimension	(2496 Frs. of SEA, 1440 Frs. of SWA)	107 (4.3)	18 (1.3)
NCI (-)	(107 Frs. of SEA, 18 Frs. of SWA)	61 (57)	10 (55)
Single peak (+)	(61 Frs. of SEA , 10 Frs. of SWA)	17 (27.87)	10 (100)

Frs.: Fractions

NCI (-): Dot blot fractions that did not react with Normal Cercaria Infection serum

Single peak (+): Dot blot fractions that have single retention peak were selected to amino acid sequencing.

Table 4: The amino acid sequences obtained from the fractions and the candidate genes with their homology and PI range .

Fraction No.	pH Range	N-terminal seq.	Identified homology	Accession No.	pI[a]
F29.2E. SEA	pH<4.80	MCVLPVD	60 CILPVD 65	AAX24607.2	4.70
			39 CVLP-- 43	AAW27155	4.60
F2.3D.SEA	8.49-8.4	MAVLPPIYKYL	3 -----PILYKYL 9	AAW27472.1	8.03
			281 MAVLP------- 285	AAW26143.1	8.02
F12.H1.SWAP	8.40 – 8.10	VPTNQN	415 -PTNQ- 418	AAW27566	8.39
			211 -PTSQN 215	AAW26607.1	8.40
F12.F1.SWAP	8.4 0– 8.10	KRRGPPGEER	131 –RRSNPPTEE- 139	AAW27690.1	8.49
			19 -----PPSEE- 23	AAX25883.1	8.49

[a] pI: has been calculated from DNAstar software (DNASTAR Inc., Madison, WI, USA).