Original article

Immunoproteomics Identification of Major IgE and IgG4 Reactive Schistosoma japonicum Adult Worm Antigens Using Chronically Infected Human Plasma

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Abstract: Immunoepidemiological studies from endemic areas have revealed age-dependent resistance correlation with increased level of IgE and decreased level of IgG4 antibodies in responses to schistosomes’ soluble worm antigen. However, there have been limited studies on analyses of major antigens that provoke IgE and IgG4 immune response during chronic stage of schistosomiasis. In this study, for the first time, immunoproteomics approach has been applied to identify S. japonicum worm antigens in liquid fractions that are recognized by IgE and IgG4 antibody using plasma from chronically infected population. ProteomeLabPF 2D fractionated 1-D and 2-D fractions of SWA antigens were screened using pooled high IgE/IgG4 reactive plasma samples by dot-blot technique. In 1-D fractions, IgE isotype was detected by fewer antigenic fractions (43.2%). The most recognized isotype was IgG3 (79.5%) followed by IgG1 (75.0%) and IgG4 (61.4%). Liquid chromatography MS/MS protein sequencing of reactive 2-D fractions revealed 18 proteins that were identified, characterized and gene ontology categories determined. 2-D fractions containing proteins such as zinc finger, RanBP2-type, domain-containing protein were strongly recognized by IgE and moderately by IgG4 whereas fractions containing proteins such as ubiquitin-conjugating enzyme and cytosolic II 5’-nucleotidase strongly recognizing by IgG subclasses (IgG1, IgG3 and IgG4) but not IgE. By this study, a simple and reproducible proteomic method has been established to identify major immunoreactive S. japonicum antigens. It is anticipated that this will stimulate further research on the immunogenicity and protective potential of proteins identified as well as discovery of novel compounds that have therapeutic importance.

Key words: Schistosoma japonicum, IgE, IgG4, Proteome, Mass Spectrometry, Genome

INTRODUCTION

The pathophysiology of schistosomiasis is mainly due to the immune response against tissue trapped eggs with consequent clinical manifestations being typical of the species infecting, intensity of worm burden as well as the immunity of the infected host. The variety of antigens released by dead worms or secreted by the worms or shed during the various developmental stages of the worm life cycle ( cercariae, schistosomula, adult male and female, and eggs) provide strong sustained stimuli to the host’s humoral and T-lymphocyte-mediated immune responses [1]. In recent years, immune response regulation by the schistosomes during infections has been a topic of great concern. Particularly, the role of antibodies in resistance to reinfection. In schistosomiasis, the balance between IgE and IgG4 antibody isotypes is thought to play a role in resistance or susceptibility to infection. Immunoepidemiological studies from endemic areas have revealed age-dependent resistance correlation with specific antibody isotype responses to the schistosome antigens, particularly IgE responses to Schistosoma mansoni adult worm antigens (AWA). The IgE levels are low in children and high in adults, whereas for IgG4 the reverse has been reported [2–4]. Furthermore, since IgE and IgG4 can exhibit parallel specificity profile, it has been suggested that IgG4 subclass acts as a blocking

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Abbreviations: BCA, Bicinchoninic acid; TCEP, Tris (2-Carboxyethyl) Phosphine Hydrochloride; n-OG, n-Octylglucoside
antibody against killing of the parasites by inhibiting IgE antibody-dependent cellular cytotoxicity (ADCC) mediated by monocytes, platelets or eosinophiles. Similar effect has also been suggested for IgM and IgG2 antibodies [2, 5–8]. The IgG3 antibody level also correlated with susceptibility to and biomarkers in liver fibrosis [6]. The production of IgE is stimulated by interleukin-13 (IL-13) and IL-4, and modulated by IL-12 and interferon-gamma (IFN-γ) while the production of IgG4 is also stimulated by IL-4 [4]. The IL-4-dependent production of IgE and IgG4 is blocked by IFN-γ, though the level required to block IL-4-dependent IgE production is much lower than that needed to block IgG4. In the sequential events of class switching, IgG4 is synthesized thereafter IgE, caused by sequential involvement of different lymphokines raising the possibility that development of protection against schistosomes would depend on population of lymphocytes producing cytokine [4, 9, 10].

In spite of many studies demonstrating importance of antibody-mediated protection against re-infection of schistosomes both in experimental and epidemiological models, many of the human schistosome vaccine research based on antibody-mediated protection have not progressed to the phase III clinical trials. This in part might be due to the limited understanding of protective anti-schistosome response against specific proteins [11]. Relatively, limited target antigens have been analyzed in the context of selective antibody isotype recognition for IgE or IgG4 especially in S. japonicum infection [2–4, 6]. Antigens that are IgE, IgG4 or both antibodies preferred can be very useful for studying mechanisms associated with antibody related resistance to schistosomiasis.

Many of the antigenic substances produced by the schistosomes at the various life cycle stages consist of proteins, glycoproteins and polysaccharides in nature [12]. So far, characterization of schistosome antigens has involved studying crude parasite extracts that had no detailed characteristics of reactive immunoglobulins. Some studies have also focused on proteins or glycoprotein components of schistosomes either directly or by cloning in bacteria systems [5, 13]. Although, elevated IgE level is important for development of resistance to reinfection in schistosomiasis, only a limited number of studies have been conducted to isolate and characterize IgE-specific antigens from S. mansoni [14] with a homologous antigen identified in S. haematobium [15] and S. japonicum [16]. Therefore, the antigenic source of variation in IgE antibody isotype-specific response to S. japonicum is limited.

The mass spectrometry (MS) based proteomics has facilitated identification of large numbers of proteins from complex biological systems. Proteomics has in recent years achieved improvements in platforms and the standard proteomics approaches rely on the second dimensional (2-D) separation of complex protein mixtures using second dimensional gel electrophoresis (2-DE) [17, 18]. In some cases, the 2-DE may be combined with difference in gel electrophoresis (DHEG) as a profiling platform and proteins are identified by ESI-MS/MS of trypsin-derived peptides. However, 2-DE has a number of shortcomings including limited loading capacity; inability to resolve proteins of extreme pI values; limitation in resolution of hydrophobic proteins and inability to resolve proteins of smaller molecular weights. Therefore, fractionating complex protein mixtures while maintaining intact proteins by liquid chromatography (LC) is most desirable for downstream analyses (top-down proteomics) [19].

The proteomeLabPF 2D instrument introduced by Beckman-Coulter (Beckman Coulter, Fullerton, CA, USA) features a rapid semi-automated 2-D HPLC system that uses two different methods to separate proteins; ion-exchange in the 1-D and non-porous reversed phase in the 2-D chromatography [20–22]. Unlike gel electrophoresis, it offers an added advantage that collected fractions are in liquid phase and can be utilized directly for any of various analytical procedures, such as enzymatic digests, mass spectrometer analysis, additional fractionation, western blot, or a combination of analytical tests. Additionally, it has been shown to be suitable for high-throughput large-scale analysis of intact proteins [23–26] and high loading capacity (up to 5 mg) than with gel electrophoresis, thus significantly increasing the sensitivity of protein identification. Liquid-based fractionation and separation systems offer great flexibility and can be suitable for large-scale proteomic profiling in a quantitative analysis [25, 26].

This study focused on isolating, identifying, and characterizing immunogenic S. japonicum proteins that are preferentially detected by IgE and IgG4 antibodies using serological proteomics approach. Identifying and characterizing antigenic proteins detected by the isotypes studied would contribute to understanding of schistosome-specific adaptive immunity. This also, highlights the importance of vaccine research focusing on induction of protective isotype-specific antibody response to specific peptides as a single protein from the parasite might possess undetermined antigenic determinants capable of stimulating various antibody productions.

**MATERIALS AND METHODS**

**Soluble Worm Antigen Preparation**

Soluble worm antigen (SWA) extract was prepared from frozen Chinese strain S. japonicum adult worms fol-
lowing the procedures previously described [27] with slight modifications. Briefly, adult worms (600 mg) were homogenized in 3.25 ml cold Diethyl Ether (Wako Pure Chemical Industries, Ltd. Osaka, Japan). The homogenate was centrifuged at 2,000 g, 5 min to remove lipids together with the diethyl ether. Thereafter, the pellet was freeze-thawed several times in 3.5 ml of lysis buffer (6 M Urea, 2 M Thiourea, 10% Glycerol, 50 mM Tris-HCl, pH 7.8, 2% n-OG, 5 mM TCEP) mixed with 0.1 mM PMSF and 2 μg/ml Leupeptin. This was dialyzed in PBS (pH 7.5) containing 8 M Urea at 4°C with stirring. The homogenate was centrifuged at 20,000 g for 1 hr at 4°C and then filtered through 0.22 μm filter (Millex GP Filter Unit, Millipore Ireland Ltd. Tullagreen, Carrigtwohill Co Cork, Ireland). Protein concentration was determined by BCA Protein Assay Kit (Bio-Rad Laboratories Inc., Tokyo, Japan) and stored at –80°C until used.

Measurement of Anti-worm Antibody Levels

An ELISA was carried out using SWA to screen plasma samples obtained from individuals with liver fibrosis (n = 31 grade 0; n = 62 grade 1; n = 91 grade 2 and 3 individuals) due to schistosomiasis japonica as previously described [28, 29]. The project proposal including the reuse of stored samples was processed to the Institutional Review Board at NEKKEN and was approved (No. 12081793). Five plasma samples originally confirmed by microscopy and ultrasound were included as positive controls. Three plasma samples were also included as negative controls which were obtained from healthy Japanese individuals without schistosomiasis history. Briefly, plates (Nunc-Immuno Plate, Nunc, Denmark) were coated with 5 μg/ml of SWA. After washing unbound antigens two times (2×) with PBS containing 0.05% Tween 20 (PBST, pH 7.4), the plates were blocked with 5% non-fat skimmed milk in PBST for 60 min at room temperature (RT) followed by 2× washing. Plasma samples were diluted 1:20 for detection of IgE and IgG4 and 1:800 for detection of IgG1 and IgG3 with 1% blocking solution at followed by incubation at 37°C for 60 min and then 3× washing. The procedure continued with 60 min incubation (37°C) with horseradish peroxidase-conjugated mouse anti-human IgG1, IgG3 (Southern Biotechnology Associates Inc., Birmingham, AL, USA), IgG4 (MP Biomedicals. Llc, France) or biotin-conjugated goat anti-human IgE (Invitrogen Corporation, Camarillo, CA, USA) in 1% blocking solution at 1:1000, 1:1000 1:400 or 1:400 respectively. For detection of IgE, the plates were further treated with 1:400 horseradish peroxidase-conjugated streptavidin (DakoCytomation, Copenhagen, Denmark). Finally, plates were developed with stabilized chromogen (SB01, Invitrogen) in the dark followed by addition of stop solution (1N H2SO4, WAKO). The OD was measured at 450 nm (iMark Microplate Absorbance Reader, Bio-Rad laboratories, Inc. Japan). The mean ODs obtained were Log-transformed after subtracting the mean ODs of the negatives and samples within the upper quartile (95 percentile) (51/184) were pooled for dot-blot reactivity against 1-D and 2-D fractionated SWA (Fig. 1A).

Buffer Exchanging and Chromatofocusing

The PD-10 desalting column containing 8.3 ml of Sephadex G-25 medium (85 to 260 μm particle size), (GE Healthcare Bio-Sciences K. K. Tokyo, Japan) was applied in buffer exchange of SWA before chromatofocusing following the manufacturer’s recommendation. Briefly, the PD-10 column was equilibrated with proprietary buffer, “ProteoSep Start” buffer (Eprogen, Darien, IL, USA) by allowing it to enter the packed bed completely. The flow-through was discarded. This was repeated with a total of 25 ml “ProteoSep Start” buffer. The 2 mg of SWA was resuspended in 1.25 ml of“ProteoSep Start” buffer, loaded onto the equilibrated PD-10 column and allowed to enter the column completely. The flow-through was again discarded. Elution was performed with 3.5 ml “ProteoSep Start” buffer added onto the column and the eluent collected into a new 15 ml tube under gravity and applied in 1-D Chromatofusing.

Chromatofusing was performed using the ProteomeLab PF 2D protein separation system (Fig. 1B) with 32 Karat user interface software. The pH gradient was formed using two proprietary buffers: “ProteoSep Start” buffer (essentially contained urea, Tris-HCl and n-OG at pH of 8.5) and “ProteoSep Elution” buffer (Eprogen, Darien, IL, USA) (essentially Urea, Polybuffer 74-HCl, n-OG and iminodiacetic acid, pH 4.0). The High Performance Chromatofusing Column (A51685 ProteoSep HPCF Column, 250 mm × 2 mm, Eprogen. Darien, IL, USA) was treated according to the manufacturer’s instructions. Briefly, the column was washed with 10 volumes of autoclaved MilliQ water at a flow rate of 0.2 ml/min for 45 min and then equilibrated with 30 volumes of “ProteoSep Start” buffer for 130 min at 0.2 ml/min, ambient temperature. The buffer exchanged SWA sample was introduced with a manual injector into the column. Proteins bound to the strong anion exchanger in the HPCF column were eluted with a continuous decreasing pH from 8.5 to 4.0. Twenty minutes after sample injection, the valve automatically switched from “ProteoSep Start” buffer to “ProteoSep Elution” buffer at a flow rate of 0.2 ml/min over 95 min. The pH began to decrease after about 45 min. Fractions were automatically collected every 0.3 pH units into a 96-well deep-plate
At 170 min, the HPCF column was washed for 45 min with 10 column volumes of a third buffer of high ionic strength solution (1 M NaCl) and re-stored by 10 column volumes of distilled water for 45 min. The absorbance of the column effluent was monitored at 280 nm with an online pH flow cell. The percentage concentration eluted over the different pH conditions was estimated using the peak area of the fraction monitored at 280 nm, a wavelength at which the peak area is directly proportional to the quantity of the proteins [30]. The 1-D fractions obtained were screened by dot-blot assay and selected reactive fractions directly applied to the 2-D reversed phase unit.

**Second Dimension Reversed Phase Chromatography**

The 2-D separation (Fig. 1D) was performed using Reversed Phase High Performance Column (391106 PF 2D HPRP Column, Beckman Coulter, Fullerton, CA, USA) and two solvents, 0.1% TFA in water (Solvent A) and 0.08% TFA in ACN (Solvent B). At the end of each run, equilibration of the column was achieved with initial mobile phase (Solvent A) for 10 min followed by Solvent B for 5 min prior to each injection. All 2-D chromatography was conducted at a column temperature of 50°C and buffer flow rate of 0.75 ml/min with the absorption of the effluent monitored at 214 nm. From the selected 1-D fractions, 200 μl was automatically injected into the PF 2D HPRP column and ran for 6 min. The column was eluted at a flow rate of 0.75 ml/min with a 0–100% linear gradient of solvent A and solvent B for 35 min. Thereafter, Solvent B was continued for 5 min, followed by re-equilibration with 100% Solvent A for 10 min. The fractions were collected at a flow rate of 0.18 min into 96-well microplate (Product code 3363, Corning International K. K. Tokyo, Japan) placed in an automated fraction collector (Gilson FC 204 Fraction Collector, M & S Instruments Inc. Osaka, Japan). The 2-D fractions were stored at –80°C while some screened by dot-blot and some.
Dot-blot Screening

The 44 1-D fractions as well as 80 2-D fractions (derived from each 1-D fraction) obtained were screened for reactivity against circulating anti-schistosome IgE, IgG4, IgG3 and IgG1 antibody isotypes in dot-blot assays in search of novel reactive proteins. The dot-blot was conducted using Bio-Dot SF Micro filtration apparatus (Bio Rad Laboratories, Inc., CA, USA) as previously described with modifications [27]. Briefly, 30 μl 1-D fraction was loaded onto polyvinylidene fluoride (PVDF) membrane (Amersham Hybond-P PVDF Membrane, GE Healthcare Bio-Sciences K. K. Tokyo, Japan) imbedded in transfer buffer (192 mM Glycine, 25 mM Tris, pH 7.4) [31] and fixed into the Bio-Dot SF Micro filtration apparatus. Following blocking, 30 μl of diluted pooled plasma (51 samples) (IgG1, 1:4000; IgG3, 1:4000; IgG4, 1:100; or IgE, 1:800) in TBS washing buffer (20 mM Tris, 137 mM NaCl, 0.01% Tween 20, pH 7.6) was applied to each respective well blotted with the fractions. Bound antibodies were incubated with respective conjugated enzymes using horseradish peroxidase-conjugated mouse anti-human IgG1, IgG3 (Southern Biotechnology Associates Inc.), IgG4 (MP Biomedicals, LLC) or biotin-conjugated goat anti-human IgE (Invitrogen) in dilutions of 1:16,000, 1:16,000 1:1,000 or 1:4,000 respectively. The IgE antibody bound membrane was further treated with 1:6,000 horseradish peroxidase-conjugated streptavidin (DakoCytomation). Blocking (5% skimmed milk/TBS washing buffer) and conjugate reaction of the membranes were conducted in a separate container. The reactivity was revealed by ImmunoStar Reagents (Wako Pure Chemicals Industries, Ltd. Osaka, Japan) for chemiluminescence detection following the manufacturer’s protocol. Digital images were obtained by the Las-4000EPUV Mini with an interface Las-4000 Image Reader (Fujifilm Corporation, Tokyo, Japan). The acquired antigenic spots were further transformed into pixels units for quantification of the recognition intensity using ATTO Lane and Spot Analyzer 6.0 software (ATTO Corporation, Tokyo, Japan). In screening of 2-D fractions by dot-blot assay, similar steps were followed. Briefly, 50 μl of each fraction in transfer buffer after drying to remove most of the ACN and TFA was applied since it was not expected for any fraction to have equal intensity by the four isotypes for determining reactivity intensity across the antibody isotypes.

In-solution Tryptic Digestion

Fractions from basic, neutral and acidic regions were treated with trypsin prior to protein sequencing. Briefly, liquid fractions (50 to 150 μl) were precipitated at ~80°C overnight in about 10-bed volume of pre-chilled acetone (Wako) followed by centrifugation at 20,000 g for 30 min at 4°C. After removing most of the supernatant, the samples were speed-vacuumed to eliminate the remaining ACN and TFA together with the acetone. Then 15 μL of denaturation solution (8 M urea; 500 mM Tris-HCl, pH 8.5; 2.5 mM EDTA) was added and incubated for 10 min at 100°C followed by cooling at RT. Addition of 5 μL of reduction solution (40 mM DTT), (Wako) in 25 mM NH4HCO3 (Wako), incubated 1 hr at 56°C with shaking followed. Alkylation reaction performed with 5 μL of 250 mM iodoacetamide (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) in 25 mM NH4HCO3 and incubated in the dark for 45 min at 25°C with shaking. A volume of 180 μL 50 mM NH4HCO3 was added to dilute out the urea and to terminate all the reactions prior to trypsin proteolytic digestion. Tryptsin proteolysis was conducted using 50 μL (10 μg/ml) sequence grade modified trypsin (Promega Corporation, Madison, WI, USA) in 50 mM NH4HCO3 overnight at 37°C followed by addition of 5 μL 5% LC-MS grade Formic Acid (Wako) in LC-MS grade ultra pure water (Wako) to terminate the reaction. The trypsinized peptides were again speed-vacuumed and resuspended in 25 μL of 0.3% formic acid before filtered in Spin-X centrifuge tubes (0.22 μm Nylon, Costar Corning Inc., Corning, NY, USA) which was spun at 2,300 g for 30 sec. The filtrate was transferred into MS vials (0.3 ml, TXP Snap Vial, GL Sciences Inc, Tokyo, Japan) for loading onto the ESI-MS/MS system.
Mass Spectrometric Analysis and Database Search

The MS and tandem-MS (MS/MS) spectra of tryptic peptides (Fig. 1E) were obtained using the NanoFrontier nLC and NanoFrontier eLD Liquid Chromatography Mass Spectrometer (Hitachi High-technologies, Tokyo, Japan). The nanoLiquid Chromatography/ElectroSpray Ionization/Linear Ion Trap/Time of Flight (nLC-ESI/LIT/TOF) and collision induced dissociation (CID) modes were used for MS detection and peptide fragmentation as described [32]. In the NanoFrontier nLC, the trypsinized peptides (1–10 μL) suspended in 0.3% formic acid were trapped on monolith trap column [C18-50-150 column, (0.05 mm I.D. × 150 mm L], Hitachi High-technologies] and separated on a packed nano-capillary column [NTCC-360/75-3-123, (0.075 mm I.D. × 100 mm L, particle diameter 3 μm), Nikkyo Technos Co., Ltd, Tokyo, Japan] at a flow rate of 200 nL/min. The peptides in the column were eluted using a stepwise ACN gradient (mobile phase A: 2% ACN, 0.1% formic acid; mobile phase B: 98% ACN, 0.1% formic acid, The A:B concentration gradient was 0.0 min: (A:B = 100:0%) → 60 min (0:100%). In the NanoFrontier eLD spectrometer, the eluted peptides were ionized with a capillary voltage of 1700 V and detected in a detector potential TOF range of 2050–2150 V.

Acquired MS and MS/MS spectra were converted into Mascot generic format (mgf) using a Data Processing software 2008 (Hitachi High-technologies) and subsequently searched in a locally established database for S. japonicum sequences downloaded from Chinese National Human Genome Centre at Shanghai (http://www.chgc.sh.cn/japonicum/Resources.html) (containing 12,657 predicted proteins) [33] using the MS/MS Ion Search provided by MASCOT Sequence Query sever version 2.3 (http://www.matrixscience.com). With the MASCOT search, the following search parameters were used, enzyme: trypsin, variable modifications: carbamidomethylation, carboxymethyl (C) and oxidation (M), mass values: monoisotopic, protein mass: unrestricted, peptide mass tolerance: ± 0.5 Da, fragment mass tolerance: ± 0.2 Da (CID data), maximum missed cleavages: 1 and Instrument type: ESI-TRAP. For MASCOT output, significant peptides were determined by the peptides score from the probability-based molecular weight search (MOWSE) which identifies proteins from the molecular weight of peptides created by the proteolytic digestion [34]. Peptide score > 27 indicate identity or extensive homology (p<0.05). Further stringency was added by eliminating any single peptide that could be assigned to more than one protein. To ensure a non-redundancy, the protein identifications were examined manually in the database for possible redundancies including multiple names and homologies.

Characterization of Identified Proteins

Identified S. japonicum proteins were characterized
according to antigenic propensity, hydrophobicity, antigenic determinant, domains and gene ontology (GO) types with respect to their antibody isotype recognition. An estimate of hydrophobicity was determined from the grand average of hydropathicity index (GRAVY) [35] using ProtParam from ExPASy [36]. The GRAVY index is the average hydropathy score for all the amino acids in the protein. The positive GRAVY index indicates hydrophobic protein and negative, hydrophilic protein. The immune peptides or antigenic determinants and antigenic propensity were also estimated [37]. With reference to conserved domain sequences (CDS) that were similar to known genes and domains, the proteins were further characterized into GO terms and annotations (biological processes, molecular functions and cellular components) using UniProt-GOA server (ver. 106) (http://www.ebi.ac.uk/QuickGO/).

Statistical Analysis

The data generated were analyzed by Microsoft Excel and GraphPad Prism ver. 5. All the data were expressed as the mean ± SD. Differences between groups were analyzed for statistical significance by one-way analysis of variance and the t-test (Unpaired t test) using the GraphPad Prism. A p-value of <0.05 was considered as statistically significant.

RESULTS

Fractions and Dot-blot Screening

Protein fractionation by chromatofocusing is used to enrich proteins with similar isoelectric point (IEP or pI) and collected in one fraction [38]. Liquid fractionation of crude SWA into 1- and 2-D was achieved using the ProtemLab PF 2D instrument. In the chromatofocusing, SWA proteins were separated and eluted into 44 fractions. Fig. 3 shows the fraction number against the elution profile (average pl) and percentage concentrations while Fig. 3, 2-D elution profiles of fractions 2, 3, 18 and 31. As expected, the basic proteins started to elute (fractions 1 to 17). The elution continued with the neutral proteins (18 to 20) followed by the weak and strong acidic proteins (21 to 44). Most of the proteins were eluted in fraction 31 (15.42%) with mainly acidic proteins. Fractions 37 to 44 had undetectable level of percentage concentration. The pl of almost all the proteins identified in the higher pH gradient were consistent with the expected pl (Table 1). However, lower pH gradient showed a weak correlation to the expected pl range even though proteins with the expected pl were also found in these fractions (F31-H7). Such unexpected phenomenon is possible because the last few fractions can be enriched with proteins that carry post-translational modifications (PTM) such as phosphorylation which can cause a shift in their pl. Usually, this phenomenon is observed in 2-D electrophoresis and is useful for identifying proteins with different modification condition [38].

Four human circulating antibody isotypes (IgE, IgG1, IgG3 and IgG4) recognition by the 1-D fractions were evaluated using dot-blot ELISA. The reactivity intensity of each fraction was transformed into pixel unit and then expressed as relative reactivity intensity. The IgE was bound by 19 fractions (43.18%) with mean intensity of 0.22 ± 0.18 while IgG1 had 33 (75.00%) with mean intensity, 0.24 ± 0.23. For IgG3 and IgG4 there were 35 (79.55%) and 27 (61.36%) fractions detected with mean intensity of 0.28 ± 0.26 and 0.36 ± 0.30, respectively. The IgE isotype was detected by fewer fractions as compared to the remaining isotypes. The most detected isotype was IgG3 followed by IgG1 and IgG4 accordingly. It was observed that fractions 2 to 6 reacted with all the isotypes with varying intensity (Fig. 3). Likewise, the 2-D fractions (2, 3, 18 and 31) were further screened by dot-blot analysis to identify wells containing proteins that were IgE or IgG4 preferred (Fig. 3). The positive dot-blot fractions of such 2-D wells were selected from basic fractions (F2-C5, F2-C7, F2-C3; F3-E1, F3-H7), neutral fractions (F18-B1, F18-C3) and acidic fractions (F31-C3, F31-E1, F31-H7) which were processed for LC-MS/ MS. Fig. 4A shows a representative 2-D dot-blot reactivity.

ESI-MS/MS Analysis

A total of 25 non-redundant proteins were identified by two-dimensional fractionation through ESI MS/MS analysis of 10 fractions (Table 1). A representative tandem mass spectrum is presented in Fig. 4B. Proteins identified with peptides obtained from the MS/MS mostly had a single confirmed peptide matches with a minimum Mascot score of 27. The 2-D wells (F2-C3, F2-C5 and F2-C7) originating from 1-D F2; 1-D F3 (F3-E1 and F3-H7); 1-D F18 (F18-B1 and F18-C3) and 1-D F31 (F31-C3, F31-E1 and F31-H7), yielded 3, 14, 4, 4 proteins respectively. Two proteins (chromatin licensing and DNA replication factor 1 and zinc finger, RanBP2-type, domain-containing) were sequenced from two sequential fractions (F2 and F3) and at the same time one of them (chromatin licensing and DNA replication factor 1), in other two non-sequential fractions (F18 and F31). Proteins with a wide range of molecular weight were identified from less than 20 kDa to more than 300 kDa (Table 1). The pl and the molecular weight profiles therefore indicate the benefit of protein fractionation which is an important aspect of protein profiling in identifying proteins with different biochemical properties. The majority of the S. japonicum proteins were recognized by IgE, IgG3 and IgG4 but not IgG1.
Characterization

It is worth noting that all the proteins identified have a GRAVY index within negative range (Table 2). Meaning the proteins are hydrophilic in consistent with 2-D reversed phase elution profile (Fig. 3). Meiosis-specific nuclear structural protein 1 (MNS1) was found to be the most hydrophilic (−1.306) and BRO1 domain-containing protein BROX, less hydrophilic (−0.177) protein. With respect to the number of possible epitope each protein have, ubiquitin-conjugating enzyme E2 N had 8 antigenic determinants, being the least yet with average antigenic propensity of 1.0386.

The GO terms for the annotated proteins in terms of biological processes, molecular function and cellular components were identified for almost all the proteins (Table S1). In terms of biological processes, some are associated with DNA metabolic and physiological processes including nucleotide metabolic process (GO:0009117), cell division (GO:0051301), mitotic cell cycle (GO:0000278), RNA interference (GO:0016246) and protein physiological processes such as potassium ion transport (GO:0006813), ATP binding (GO:0005524) and protein modification process (GO:0006464). Others were associated with regulation, development or stress response including cell differentiation (GO:0030154), reproduction (GO:0000003) and response to oxidative stress (GO:0006979) and so on. In terms of cellular components, the proteins were found to be associated with cytoplasm (GO:0005737), intracellular (GO:0005622), nucleus (GO:0005634), plasma membrane (GO:0005886) and extracellular region (GO:0005576). The PDZ and LIM domain protein 3 expressed in the cytoplasm is associated with response to oxidative stress (GO:0006979). In addition, a membrane protein, Small conductance calcium-activated potassium channel (SK) protein 2 with Calmodulin binding domain (CaMBD) (GO:0015269), has been found to be a secreted protein [39–42].

Fig. 3. Second dimensional elution profile of 1-D fractions 2 (A), 3 (B), 18 (C) and 31 (D). 1-D fractions were run in the 2-D reversed phase chromatography using the ProteomeLab PF 2D. As shown is the elution profile with respect to time and absorbance units (AU). The elution gradient was achieved using two solvents, 0.1% TFA in water (A) and 0.08% TFA in ACN (B). The 2-D fractionation was run at column temperature of 50°C and a buffer flow rate of 0.75 ml/min with the absorption monitored at 214 nm. The column was eluted with a 0–100% linear gradient of solvent A and B for 35 min. Insert, 2-D UV difference maps obtained by ProteoVue of the fractions and the pH range; 31 (pH 4.29–4.34), 18 (pH 6.03–6.33), 3 (pH 8.22–8.45) and 2 (pH 8.45–8.47). Arrows indicate fractions from which proteins were sequenced. F2 P# = 14, 23, 29. F3 P# = 14, 23, 29. F18 P# = 3/4, 13. F31 P# = 6, 16, 47. P#, peak numbers.
Table 1. Fractions and identified proteins by ESI MS/MS. *S. japonicum* adult worms extract was liquid fractionated using ProteomeLab PF 2D system into 1-D by chromatofocusing and reversed phase chromatography followed which were screened by dot-blot. Immunoreactive 2-D fractions were subjected to ESI MS/MS and peptide identification using MASCOT server with a locally established *S. japonicum* protein sequences downloaded from *S. japonicum* database at http://www.chgc.sh.cn/japonicum/Resources.html. MP, MASCOT peptides score. *Total MASCOT Peptide Score.

| Fraction | Well | Protein ID Match to | Unique peptide | m/z | z error (Da) | Peptide | MP Score | pI/Mw |
|----------|------|---------------------|----------------|-----|--------------|---------|----------|-------|
| F2-C3    | Sjc  | _0203170_ Chromatin licensing and DNA replication factor 1 | 1 | 530.81 | 2 | 0.0092 | DVIDLVKMK | 56 | 9.74/64520 |
| F2-C5    | Sjc  | _0213700_ Zinc finger, RanBP2-type, domain-containing | 1 | 450.29 | 2 | 0.0517 | INLSSLPR | 27 | 9.34/57512 |
| F2-C6    | Sjc  | _0213700_ Zinc finger, RanBP2-type, domain-containing | 1 | 450.29 | 2 | 0.0503 | INLSSLPR | 30 | 9.34/57512 |
| F3-E1    | Sjc  | _0034740_ Meiosis-specific, nuclear structural protein 1 | 1 | 689.85 | 2 | -0.0309 | RELEAINAYTAK | 40 | 6.79/45874 |
| F3-E1    | Sjc  | _0203170_ Chromatin licensing and DNA replication factor 1 | 1 | 530.80 | 2 | -0.0808 | DVIDLVKMK | 36 | 9.74/64520 |
| F3-E1    | Sjc  | _0058190_ NACHT and WD repeat domain-containing protein 1 | 1 | 590.33 | 2 | 0.0613 | MCEQLLKTR | 34 | 5.28/228171 |
| F3-E1    | Sjc  | _0037420_ Triple functional domain protein | 1 | 606.37 | 2 | 0.0134 | QFLAK | 30 | 6.13/73854 |
| F3-E1    | Sjc  | _0086690_ RNA Helicase | 1 | 606.37 | 2 | 0.0465 | MQGLK | 30 | 9.07/86337 |
| F3-E1    | Sjc  | _0041010_ Protein kinase | 1 | 699.86 | 2 | 0.0254 | ENFVLDEIEK | 29 | 8.86/20937 |
| F3-E1    | Sjc  | _0302250_ BRO1 domain-containing protein BROX | 1 | 564.33 | 2 | 0.0496 | EKAGQAIAR | 31 | 8.28/47921 |
| F3-E1    | Sjc  | _0111110_ Cell division control protein CDC7 | 2 | 464.79 | 2 | 0.1187 | LIPECEKE | 32* | 9.5/36413 |
| F3-E1    | Sjc  | _0061880_ Centrin | 3 | 572.88 | 2 | 0.1375 | GELEFIKAE | 51* | 7.04/27899 |
| F3-E1    | Sjc  | _0064400_ Conductance calcium-activated potassium channel protein 2 | 2 | 481.32 | 2 | 0.1387 | FISLCNHK | 31* | 7.09/169207 |
| F3-E1    | Sjc  | _009730_ CREB-binding protein | 2 | 679.52 | 2 | 0.1672 | NLVTSMGVLSGYMPR | 32* | 8.00/113622 |
| F3-E1    | Sjc  | _0164990_ PDZ and LIM domain protein 3 | 3 | 564.31 | 2 | 0.0968 | VPMHEPCLK | 30* | 9.08/35351 |
| F3-E1    | Sjc  | _0054640_ Prolyl-tRNA synthetase | 3 | 488.93 | 2 | 0.1187 | KTGQOGLRCCVR | 31* | 7.5/128901 |
| F3-E1    | Sjc  | _0213700_ Zinc finger, RanBP2-type, domain-containing | 1 | 450.29 | 2 | 0.0415 | INLSSLPR | 30 | 9.34/57512 |
| F3-E1    | Sjc  | _0058190_ NACHT and WD repeat domain-containing protein 1 | 1 | 590.33 | 2 | 0.0763 | MCEQLLKTR | 31 | 5.28/228171 |
DISCUSSION

Following skin penetration by cercariae, *S. japonicum* adult worms migrate to the hepatic portal system, where they mature and survive for many years where the female occasionally migrating to the smallest venules to lay eggs [43]. The adult schistosomes are constantly exposed to the host immune system with antibodies produced against fractions of the worms. These antibodies are often used as potential diagnostic tools. Several immunoepidemiological studies have examined antibody isotype responses to schistosomal protein extracts in the form of isolated proteins, recombinant proteins or crude antigen [44, 45]. Many studies have also presented a global proteomics approach using 2-D gels to identify major *S. japonicum* excretory and secretory proteins as well as adult worm and egg extracts [46–50]. In this study, for the first time, proteomics approach was extended to identifying *S. japonicum* proteins in ProteomeLab FP 2D derived liquid fractions reactive to antibody isotypes in plasma samples from *S. japonicum*-infected population. ESI MS/MS was applied to sequence fractions containing immunoreactive proteins. In all, 18 proteins were identified; characterized and GO categories determined to enhance understanding of the immunological significance of these proteins.

In IgE, IgG1, IgG3 and IgG4 antibody isotype recognition of 1-D fractions, it has been shown that some fractions from the adult *S. japonicum* proteome are preferentially recognized by certain isotypes. For instance, IgE isotype was detected by fewer antigenic fractions (43.2%). The most recognized isotype was IgG3 (79.5%) followed by IgG1 (75.0%) and IgG4 (61.4%) accordingly. The IgG3 response was directed against a larger repertoire of antigens in the fractions. This suggests that there were fewer dominant antigens stimulating IgE response. Earlier report [51, 52] showed where IgE reactivity to glycolipids extracted from schistosome eggs (SEA) or adult worms (SWA) was more than IgG4 and that proteins alone do not constitute the major binding targets of IgE, and that this isotype is substantially directed towards carbohydrate moieties portion of glycolipids on proteins present in SEA or AWA. Weiss *et al.* [53] showed that a carbohydrate epitope recognized by a monoclonal antibody that was raised against the cercarial glycocalyx was present on glycoproteins and glycolipids of various schistosomes’ life cycle stages. This might explain why IgG4 recognized more proteins than IgE in the 1-D dot-blot.

Furthermore, zinc finger, RanBP 2-type, domain-containing protein was strongly recognized by IgE but moderately by IgG3 and IgG4 and weakly by IgG1 indicating that it might play less role in IgG subclasses directed immune response. The antigens recognized strongly by IgE are of interest as such antigens could be associated with development of resistance to schistosomiasis [2–4, 6]. The E3 ubiquitin-protein ligase, ubiquitin-conjugating enzyme E2 N and 5’-nucleotidase, cytosolic II were strongly recognizing by IgG subclasses (IgG1, IgG3 and IgG4) but not IgE suggesting these enzymes might be of IgG subclasses preferred. The serine/threonine-protein kinase with a relatively higher antigenic propensity of 1.0118 was sequenced from a single 2-D well strongly reactive with all the four isotypes indicating strong preference for IgE, IgG1, IgG3 as well as IgG4 suggesting shared antigenic determinants or multiple epitopes. This highlights the importance of vaccine research.

**Fig. 4.** Representative 2-D fraction dot-blot reactivity and tandem mass spectrum. Represented by A, IgE reactivity intensity of F2-C3, F2-C5 and F2-C7 with crude SWA and BSA controls. B, a tryptic INLSSLPR peptide of Zinc finger, RanBP2-type, domain-containing protein, Sjc_0213700 (GenBank: CAX74641.1). The precursor ion was m/z 450.30(2+). Sjc_0213700 was sequenced from F2-C5 and F2-C7 with strong preference for IgE.
Table 2. Biochemical properties and immunoreactivity pattern of the proteins. Dot-blot assay was performed for each second dimension fraction containing the proteins identified. The reactivity was quantified into pixels unit and graded according to reactivity intensity with respect to that of the crude parasite antigen to obtain relative reactive intensity. Using the relative reactive intensity, each reactive spot was scored as 'weak reactivity' (−), 'moderate reactivity' (±) or 'strong reactivity' (+). In addition to reactive pattern, proteins were characterized using GRAVY index (ProtParam tool), number of antigenic determinant contained in the full amino acid length and the antigenic propensity where >1 indicates high antigenic character. In this Table antibody reactivity pattern is not repeated for proteins from the same fraction well.

| Fraction | Well | Protein identified | GRAVY index | Amino acids | Antigenic determinants | Antigenic propensity | Antibody reactivity pattern |
|----------|------|--------------------|-------------|-------------|------------------------|----------------------|----------------------------|
| F2-C3    |      | Chromatin licensing/DNA replication factor 1 | –0.348      | 578         | 20                     | 1.0414               | + – – +                   |
| F2-C5    |      | Zinc finger, RanBP2-type, domain-containing | –0.986      | 513         | 21                     | 1.0108               | + – – ±                   |
| F2-C7    |      | Zinc finger, RanBP2-type, domain-containing | –0.986      | 513         | 21                     | 1.0108               | ± – – ±                   |
| F3-E1    |      | Meiosis-specific, nuclear structural protein 1 | –1.306      | 376         | 11                     | 0.9936               | + ± + +                   |
|          |      | Chromatin licensing/DNA replication factor 1 | –0.348      | 578         | 20                     | 1.0414               | + ± + +                   |
| F3-H7    |      | Triple functional domain protein | –0.414      | 1554        | 68                     | 1.0336               | – ± – ±                   |
|          |      | RNA Helicase [EC:3.6.1.-] | –0.409      | 762         | 25                     | 1.0308               | + – – –                   |
|          |      | Protein kinase [EC:2.7.1.-] | –0.22       | 1921        | 63                     | 1.0397               | – ± + +                   |
|          |      | BRO1 domain-containing protein BROX | –0.177      | 426         | 16                     | 1.0459               | – ± + +                   |
|          |      | Cell division control protein CDC7 | –0.333      | 330         | 13                     | 1.0363               | + ± + +                   |
|          |      | Centriolin | –0.847      | 2444        | 92                     | 1.0139               | – ± + +                   |
|          |      | Small conductance calcium-activated potassium channel protein 2, putative | –0.393      | 1536        | 58                     | 1.0294               | – ± + +                   |
| F3-H7    |      | CREB-binding protein | –0.708      | 986         | 29                     | 1.0269               | – ± + +                   |
|          |      | PDZ and LIM domain protein 3 | –0.674      | 317         | 13                     | 1.0199               | – ± + +                   |
|          |      | Prolyl-tRNA synthetase [EC6.1.1.15] | –0.473      | 1135        | 41                     | 1.0295               | – ± + +                   |
| F3-H7    |      | Zinc finger, RanBP2-type, domain-containing | –0.986      | 513         | 21                     | 1.0108               | – ± + +                   |
| F3-E1    |      | E3 ubiquitin-protein ligase HUWE1 | –0.423      | 2720        | 105                    | 1.0231               | – ± + +                   |
|          |      | 5'-nucleotidase, cytosolic II | –0.2        | 476         | 21                     | 1.0368               | – ± + +                   |
| F3-H7    |      | Chromatin licensing/DNA replication factor 1 | –0.348      | 578         | 20                     | 1.0414               | – ± + +                   |
|          |      | Ubiquitin-conjugating enzyme E2 N | –0.228      | 173         | 8                      | 1.0386               | – ± + +                   |
| F31-C3   |      | Chromatin licensing/DNA replication factor 1 | –0.348      | 578         | 20                     | 1.0414               | – ± + +                   |
| F31-E1   |      | Putative serine/threonine-protein kinase C05D10.2 | –0.774      | 1061        | 39                     | 1.0118               | + ± + +                   |
| F31-H7   |      | Chromatin licensing/DNA replication factor 1 | –0.348      | 578         | 20                     | 1.0414               | – ± + +                   |
|          |      | NACHT/WD repeat domain-containing protein 1 | –0.282      | 2005        | 81                     | 1.0271               | – ± + +                   |
focusing on induction of protective isotype-specific antibody response to specific peptides since a single protein from the parasite might possess undetermined antigenic determinants capable of stimulating various antibody productions. Therefore, further investigations employing peptide mapping techniques will be essential in determining specific antigenic determinants for the isotypes.

There were also proteins found with strong immunogenic activity to IgE, IgG3 and IgG4 but not IgG1. Some of these proteins were the Small Conductance calcium-activated potassium channel protein 2, BRO1 domain-containing protein BROX both of which are membrane associated proteins and PDZ/LIM domain containing protein 3. Notwithstanding, it is possible that such proteins might have multiple isotype-specific or shared antigenic determinants within each protein. The PDZ/LIM domain containing proteins are known to be both interaction modules associated with proteins of diverse functions [20, 54].

Proteins associated with nucleus, or cytosolic component are not immediately and directly exposed to the immunity of the definitive host and hence might not evoke immune response. However, Harn, et al. [55], identified glycolytic enzymes, triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase to be expressed on the surface of larval schistosomes and it appears very unlikely that these fundamentally cytosolic proteins are exposed at the external surface of the parasites. However, evidence from other cell systems showed that both glycolytic enzymes and various heat shock proteins can bind to cytoskeletal structures such as actin, microfilaments and microtubules [56, 57] where they can evoke immune response from the host. In addition, small conductance calcium-activated potassium channel (SK) protein 2 with calmodulin binding domain (CaMBD), a membrane associate protein that can also be secreted has been reported to function in host-parasite interaction [39, 41, 42].

In this study, attempt was made to identify and characterize potential IgE and IgG4 immunoreactive proteins employing immunoproteomics approach and related identified proteins with their biochemical properties and gene ontology types. A number of proteins were sequenced from immunoreactive fractions. It is anticipated that, immunoreactive proteins identified herein will stimulate further studies to evaluate their immunogenicity through recombinant protein expression, immunomoderation properties in terms of protective potentials and novel compounds that have therapeutic importance.

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