3. MATERIALS AND METHODS

3.1 Preparation of *Biomphalaria* and *Schistosome* Material

B. glabrata snails were used to maintain four species of *S. mansoni*, a Brazilian, Egyptian, Kenyan and Puerto Rican strain, in the laboratory. The snails were kept in 15 litre aquarium tanks filled with tap water which was allowed to dechlorinate by being left to stand for 24 hours, also allowing temperature equilibrium. It was supplied through non-copper piping. The room was maintained at 26-28^{o}C and illuminated on a 12 hour cycle with 'Warm White' fluorescent tubes. The snails where fed daily with commercial pelleted rabbit food, and the water was supplemented with Nolan-Carriker salt solution (Malek & Cheng, 1974) at a concentration of 1 ml per litre. The tanks were aerated by airstones connected to a constant air supply.

Snails were infected with miracidia obtained from eggs in infected mice liver. To infect the snails, hatched miracidia were added to a tank containing between 50-100 snails in 1-2 litres of water at 26 ^{o}C and left undisturbed overnight. The following day the procedure was repeated. The snails where placed into a full tank of fresh water with Daphnia, hardener salts and aeration after a further 24 hours. After 30 days the snails were screened to determine whether they were infected or not. Infected snails were separated from uninfected snails by putting individual snails in glass tubes. Uninfected snails were left in the tank for a further week and screened again to see whether infection by the miracidia had taken place, uninfected snails were discarded.

The hepatopancreases of infected snails were harvested and placed into protease inhibitor (Sigma, UK) and Phenylmethylsulfonyl fluoride (PMSF) (Sigma, UK) to prevent uncontrolled proteolysis. The samples were 'snap frozen' in liquid nitrogen and stored at -80 ^{o}C.

The protein samples were homogenised using a lysis buffer I (Appendix 1) containing urea and thiourea which solubilises the proteins.

3.2 Removal of Contaminants

A Perfect-FOCUS kit (GENOTECH St. Louis, MO USA) was used to concentrate and remove contaminants from the crude hepatopancreas samples, according to manufacturer's instructions (Appendix 2), in preparation for 2DE. This process removes interfering compounds such as lipids, polysaccharides, nucleic acids, salts and proteases.

3.3 Protein Estimation

Protein estimation took place post Perfect-FOCUS using Bradford reagent (Sigma-Aldrich, UK) according to manufacturer's instructions, based up on the method of Bradford (1976). All samples were assayed upon a Cary 50 Bio UV-visible spectrophotometer, set at a wavelength of 595 nm. An equal volume of buffer in which proteins were dissolved was added to blank controls to account for any interaction between buffer components and Bradford Reagent (Sigma-Aldrich, UK) . Bovine serum albumin was used as a calibration standard. The sample from

susceptible snails contained 2.95 μg/μl and the sample from resistant snails contained 2.22 μg/μl.

3.4 First Dimension Isoelectric Focusing (IEF)

IEF, the first stage of 2DE, separates proteins by charge or p*I*. 500 μg of protein sample was added to lysis buffer II (Appendix 3) to bring the total sample volume up to 300 μl, the maximum volume of protein and lysis buffer solution for loading on to the strips.

The strips used where BioRad ReadyStrip IPG Strips, Non linear 17 cm pH 3-10. The isoelectric focusing step involved active rehydration of the sample, as apposed to passive rehydration, as larger protein samples can be used, and it is considered more reliable. This involves the gel strip being rehydrated in the presence of the sample under a low voltage electric current. Wicks were wet with ddH_2O and placed on electrodes in the well of the tray. The sample was added equally along the full length of the well, the strip was laid gel side down in the well, poles matching, ensuring no air bubbles were trapped beneath the gel. The strip and sample was covered in mineral oil preventing evaporation. The lid was placed over the tray, and the IEFCell system™ (BioRad) programme run (Appendix 4).

3.5 Equilibration of Strips

Before SDS-PAGE (sodium dodcyl sulphate polyacrylamide gel electrophoresis), it was necessary equilibration of the IEF strips to allow reduction and alkylation of the proteins to take place, and give them a negative charge. Strips

were removed from sample well using forceps to hold plastic on negative end, washed briefly in ddH_2O in petri dish (gel side facing inwards – away from outside of petri dish). 3 µl of equilibrium buffer I (Appendix 5) was applied to the strips for 15 minutes and placed on a rocking platform. The strips were then washed with double distilled water before 3 µl of equilibrium buffer II (Appendix 6) was applied to the strips for a further 15 minutes, allowing carbamidomethylation of the proteins.

3.6 Second Dimension – SDS – PAGE

SDS-PAGE, the second step in 2DE, allows separation of proteins by their MW. The SDS-PAGE equipment was set up according to the method of LaemmLi (1970).

A 12.5% T, 3% C resolving gel (Appendix 7) was poured, overlayed with a 3% T, 3% C stacking gel (Appendix 8). Molten low melting point agarose (Appendix 9) was added, into which gel side facing out, IEF strips were quickly loaded, creating the polyacrylamide gel. An electrode wick containing 5 µl of Amersham low molecular weight markers (Amersham Biosciences, UK), was placed at the positive side of the gel.

The gels were locked into the tank, and then TGS buffer was used to fill the upper and lower chamber. The proteins were separated at 100 V for an hour, the voltage was then increased to 200 V for five hours until the dye front had migrated to the terminal position 25 mm from the bottom of the gel. Once complete gels were stored in fixative.

3.7 Staining the Gels

In order to visualise the proteins in the gels Coomassie blue (Appendix 10), capable of detecting 20-40 ng protein per spot/band, was applied overnight. After destaining the gels (Appendix 11) it was apparent that Coomassie blue was not sensitive enough for the image analysis. Silver stain (Appendix 12), capable of detecting 0.5-1.2 ng protein per spot/band was then applied allowing more spots to be visualised. Both stains were used according to manufacturer's guidelines.

3.8 Scanning of Gels

Gels were scanned into a GS-800 Calibrated Densitometer (Bio-Rad, Hemel Hempstead, Hertfordshire, UK), using the software Quantity One (BioRad, UK) saving the image as TIF (tagged image files) for image analysis.

3.9 Image Analysis of Gels

To analyse the two gels the computer software package Progeneis PG220 (Nonlinear Dynamics Ltd., UK) was used.

The 2006 algorithm for spot detection was used and the spot pattern manually edited. A reference gel was then created from the gel containing the highest number of spots (Gel from the resistant snails) and all the spots in the slave gels matched to it, using both manual and automatic matching. Following background subtraction, normalised spot volumes were calculated for each spot. Each automated analytical step was manually edited. Comparisons were then created between the two gels which

provided data regarding the up-regulation and down-regulation of spot and allowed the identification of unique spots.

3.10 Trypsin in Gel Digestion of Proteins

Protein spots of interest from Baker's (unpublished, 2008) and Murray's (unpublished, 2008) gels (spots 201 and 594) were manually excised from the stained 2DE gels using 1.5 mm pen cutters, and the resulting gel plugs were washed in fresh water. Protein plugs were then subject to in gel digestion (Appendix 13) to yield peptide fragments for mass spectrometric analyses.

Due to poor results an additional protein spot (885) was manually excised and subject to an alternative protocol for in gel digestion (Appendix 14) as well as the original protocol (Appendix 13). When new gels were run spots 1880, 1906 and 2017 were excised and subject to the second protocol for in gel digestion (Appendix 14).

3.11 Tandem Mass Spectrometry (ESI-MS/MS) of Peptides

For tandem mass spectrometry (MS/MS) a Q-ToF 1.5 Mass Spectrometer was used. All Q-ToF MS/MS was carried out by Jim Heald at Aberystwyth University. Fragmentation spectra from MS/MS were interpreted directly using Peptide sequencing, MassLynx v 3.5. All acquired spectra were combined to produce a single fragmentation spectrum. Each fragmentation spectrum was processed by smoothing, background subtraction and finally processing with MaxEnt deconvolution software (MassLynx v 3.5).

Sequence interpretation was initially conducted automatically using peptide mass sequencing within MassLynx v 3.5. During analyses three common protein modifications were allowed including carbamidomethylation and acrylamide of cysteine residues and oxidisation of methionine residues. When automatic sequence interpretation did not yield good results, peptides were sequenced manually. Trypsin was specified as the enzymatic choice to generate peptide fragments. Peptide sequence BLAST searches were conducted upon the metazoans of the NCBI non-redundant database (available at http://www.ncbi.nlm.nih.gov/).

3.12 Peptide Mass Fingerprinting (PMF)

Monoisotopic peptide masses were recorded (Appendix 15) from the calibrated spectra for each of the samples using MassLynx V3.5 (Micromass Ltd., UK). PMF searches were undertaken within the publically available MASCOT search tool, software version 1.8, and metazoans of the NCBI non-redundant database provided by Matrix Science (http://www.matrixscience.com, Matrix Science Ltd., UK). Trypsin was specified as the enzymic choice to generate peptide fragments. Only one maximum missed cleavage was allowed with a peptide mass tolerance of 1.2 Da. For reliable identification through MASCOT several factors were used to assign a significant identification. Expectancy value (E-value), MOWSE (molecular weight search) score, % coverage, theoretical MW and theoretical pI were noted. E-values are defined by MASCOT as the number of times expected to obtain an equal or higher score, purely by chance. Therefore, the lower this value, the more significant the result. The MOWSE scoring algorithm is described by Pappin, Horjrup & Bleasby (1993). MASCOT incorporates a probability based implementation of the MOWSE

algorithm, which accurately models the behaviour of a proteolytic enzyme. Positive protein identification was assumed from results providing significant (at the 5% level) probability based MOWSE scores.