**Purification of a chymotrypsin-like enzyme present on adult
Schistosoma mansoni worms from infected mice and its
characterization as a host carboxylesterase**

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Short running title:

A host-derived enzyme found on the surface of *S. mansoni* worms.

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SUMMARY

A serine protease-like enzyme found in detergent extracts of *S. mansoni* adult worms perfused from infected mice has been purified from mouse blood and further characterized. The enzyme is approximately 85 kDa and hydrolyses N-acetyl-DL-phenylalanine β -naphthyl-ester, a chromogenic substrate for chymotrypsin-like enzymes. The enzyme from *S. mansoni* worms appears to be antigenically and enzymatically similar to a molecule that is present in normal mouse blood and so is seemingly host-derived. The enzyme was partially purified by depleting normal mouse serum of albumin using sodium chloride and cold ethanol, followed by repeated rounds of purification by one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1-D SDS-PAGE). The purified material was subjected to tandem mass spectrometry and its derived peptides found to belong to mouse carboxylesterase 1C (CES1C). Its ability to hydrolyse α - or β -naphthyl acetates, which are general esterase substrates, has been confirmed. A similar carboxylesterase was purified and characterized from rat blood. Additional evidence to support identification of the enzyme as a carboxylesterase has been provided. Possible roles of the enzyme in the mouse host-parasite relationship could be to ease the passage of worms through the host's blood vessels and/or in immune evasion.

Key words: *S. mansoni*, host-derived enzyme, mice, carboxylesterase 1C, immune evasion

KEY FINDINGS

- A host-derived enzyme found in detergent extracts of adult *S. mansoni* worms from infected mice has been purified.
- Physical and biochemical characteristics of the enzyme enabled its characterization as a carboxylesterase.
- The purified enzyme hydrolysed two esterase substrates: β -naphthyl and α -naphthyl acetates.
- The purified enzyme was inhibited by a serine protease inhibitor and a carboxylesterase inhibitor.
- The host-derived carboxylesterase could play a role in host physiology and/or immune evasion.

83 INTRODUCTION

84 Schistosomes are long-lived parasitic flat worms inhabiting host vasculature.
85 Five species out of twenty are able to infect man, establishing a chronic infection
86 (Standley *et al.*, 2012). One immune evasive strategy employed by the parasite and
87 presumed to prolong its survival is the acquisition of host molecules. Several host
88 molecules have been reported on the surface of *Schistosoma mansoni* adult worms,
89 including blood group antigens (Smithers *et al.*, 1969), major histocompatibility
90 complex antigens (Simpson *et al.*, 1986), immunoglobulins (Kemp *et al.*, 1976), host
91 lipids (Furlong *et al.*, 1992), lipoproteins (Dingirard and Yoshino, 2006), alpha-2-
92 macroglobulin (Damian *et al.*, 1973), contrapsin (Modha *et al.*, 1988) and
93 components of the complement system (Skelly, 2004).

94 A relationship between a host-derived chymotrypsin-like serine protease
95 present in detergent extracts of *S. mansoni* worms that had been perfused from
96 infected mice and also in mouse blood was previously reported by Darani and
97 Doenhoff (2008). The enzyme was found in a detergent (deoxycholic acid - DOC)
98 extract of worms with relatively little being found in non-detergent extracts and it was
99 seemingly identical antigenically and enzymatically to the molecule in mouse serum.
100 The antigen in the worm extract and normal mouse serum were both
101 immunoprecipitated in immunoelectrophoresis by a rabbit antiserum raised against
102 whole mouse serum (anti-NMS) (Darani and Doenhoff, 2008). The enzyme activity
103 was visualized using N-acetyl-DL phenylalanine β -naphthyl ester (NAPBNE), a
104 chromogenic substrate of chymotrypsin-like enzymes. The molecule in both the DOC
105 worm extracts and NMS is of interest because it is unusual for an enzymatically-
106 active form of a protease to be present in blood - most are there as inactive pro-
107 enzymes or in zymogen form. Furthermore, while the active enzyme has been found

in mouse and rat blood, it was not found in the blood of several other mammalian species including sheep, cattle and humans (Darani and Doenhoff, 2008).

This study reports the purification and characterization of the mouse plasma-derived chymotrypsin-like enzyme, which we believe to be identical to that found on the surface of *S. mansoni* worms recovered from infected mice. Mass spectrometry helped determine the amino acid sequence of the enzyme which in turn enabled it to be identified and further characterized. Information so derived gave insights into its possible role in the host parasite relationship.

MATERIALS AND METHODS

All chemicals and buffers were of analytical grade and bought from Sigma-Aldrich Company limited, UK, except when otherwise stated. These included absolute ethanol, sodium acetate, the salt constituents of phosphate buffered saline (PBS), Tris, triton-X100, N, N, N-acetyl-DL-phenylalanine β -naphthyl ester (NAPBNE), fast blue B salt (FBB), β -naphthyl acetate, α -naphthyl acetate, fast red TR, dimethyl sulfoxide (DMSO) and glycine.

Parasite extract and antigens

A Puerto Rican isolate of *S. mansoni* was routinely used for infection of mice for experimental work and production of adult worm antigens. The isolate has been maintained by continuous passage in random-bred mice and *Biomphalaria glabrata* snails, the intermediate host for the generation of cercaria. Adult schistosomes were recovered by portal perfusion of infected mice 42 days after infection, as described originally by Smithers and Terry (1965) and modified by Doenhoff *et al.* (1978).

Detergent extracts of *S. mansoni* worms (WM) were prepared by re-suspending freshly perfused, gravity-sedimented worms which had had all visible traces of erythrocytes removed from the suspending fluid, in twice their volume of 2% deoxycholic acid (detergent) solution in isotonic saline as previously described by Doenhoff *et al.* (1988). The suspension was gently agitated for 4 hours at room temperature and then centrifuged. The supernatant was removed and stored at -80°C until used for rabbit immunization or in immunoprecipitation studies.

151

152 *Normal mouse serum (NMS) and normal rat serum (NRS)*

153 Normal mouse serum and normal rat serum were prepared by exsanguinating
154 healthy uninfected animals of the respective species. Collected blood was put into
155 universal tubes and stored in a fridge at 4⁰C for 4 hours to clot. The blood clots were
156 ringed with a pipette to aid separation of the clot from serum and the serum
157 removed, centrifuged at 2,500 x g for 6 minutes at 4⁰C and the clear supernatant
158 removed and stored at -80⁰C until use.

159

160 *Rabbit anti-normal mouse serum (Anti-NMS)*

161 A polyspecific rabbit anti-normal mouse serum antiserum was prepared as
162 described by Darani and Doenhoff (2008) by repeated weekly injections of 1 ml
163 emulsion containing equal volumes of normal mouse serum and Freund's adjuvant.
164 The response was assessed qualitatively in terms of the intensity of immuno-
165 precipitation lines yielded by the serum and the homologous antigen extract. The
166 rabbit was serially bled weekly from alternate ears until enough serum was collected.
167 The serum pool was divided into 5 ml aliquots and stored at -20⁰C.

168

169 *Rabbit anti- complete Freund's adjuvant (Anti-CFA)*

170 Rabbit anti-CFA was prepared as described for anti-NMS except that rabbits were
171 injected only with repeated weekly doses of 1 ml emulsion of CFA.

172

173 *Rabbit anti-mouse serum albumin (anti-MSA)*

174 A rabbit anti-mouse serum albumin antiserum was raised by immunization
175 with replicate immunoprecipitin arcs produced by immunoelectrophoresis of mouse
176 serum albumin and anti-NMS as described by Goudie *et al.* (1966), adapted as in
177 Dunne *et al.* (1986). Immunoprecipitin arcs were excised, homogenized and injected
178 into rabbits at weekly intervals. Antibody responses of Immunized rabbits were
179 monitored and finally bled as described for the rabbit anti-NMS.

180

181 *Rabbit anti-worm protease antiserum*

182 A rabbit antiserum specific for the chymotrypsin-like enzyme in NMS was
183 prepared as described by Darani and Doenhoff (2008).

184

185 *Reduction of albumin in NMS and NRS*

186 Serum albumin was depleted from both NMS and NRS as described by
187 Colantonio *et al.* (2005). NaCl was added to a known volume of each serum in a
188 micro-tube to give a final concentration of 0.1 M and the mixture incubated with
189 gentle rotation for 60 minutes at 4⁰C. Cold ethanol was added to the mixture to yield
190 a final concentration of 42% v/v and incubated for a further 60 minutes at 4⁰C. The
191 mixture was centrifuged at 16000 x g for 45 minutes at 4⁰C and the resultant
192 supernatant transferred into a sterile micro tube 'B' for further processing while
193 retaining the pelleted precipitate in the first tube 'A'. The pH of the supernatant was
194 lowered to 5.7 by adjusting with cold 0.8 M sodium acetate, pH 4.0 and incubated at
195 4⁰C for 60 minutes. The mixture was centrifuged as above and supernatant

containing mainly albumin transferred into sterile micro-tube 'C' while retaining the pelleted precipitate. The first and second precipitates were separately reconstituted with 0.1 M PBS, pH 7.4 (weight/volume) and stored at -20°C. The protein concentration in NMS was 23.70 mg/ml while concentrations in solutions of the two precipitates A and B and the supernatant were 5.50, 5.90 and 10.90 mg/ml respectively.

Immunochemistry for the detection and purification of the chymotrypsin-like enzyme

Single radial immunodiffusion (RID) (Mancini *et al.*, 1965) was carried out as described by Darani *et al.* (1997), on microscopic glass slides using 1% molten agarose in 0.06 M barbitone buffer, pH 8.6. The immuno-precipitate was washed in several changes of 0.9% saline to remove non-immunoprecipitated material.

Purification of the chymotrypsin-like enzyme in mouse and rat sera was achieved in one-dimensional sodium dodecyl 12% acrylamide gel electrophoresis (Laemmli, 1970) as modified by Studier (1973). 18 ug of each serum (mouse or rat) were loaded into separate gels with broad wells (6.2 cm long) in replicates and electrophoresed. Bands with enzymatic activity were excised from replicate thin strips of the polyacrylamide gel, put into a 1.5 ml Eppendorf container and covered with a minimum volume of elution buffer (0.06 M Tris-HCl, 10% SDS, pH 7.0) (Beyer *et al.*, 2008). The tube was incubated at 37°C for 24 hours, centrifuged at 14,000 x g for 30 minutes at the same temperature and the resultant eluate was removed. Further purification of the enzyme was achieved by re-electrophoresing the eluate in a fresh SDS-PAGE gel. The whole sequence of (i) SDS-PAGE, (ii) elution of enzyme activity from gel strips, (iii) re-electrophoresing in PAGE was repeated three times in

an effort to obtain a sufficiently pure sample of the enzyme suitable for analysis by mass spectrometry (MS). PAGE gels carrying proteins for analysis in MS were stained using SimplyBlue SafeStain (Invitrogen, Carlsbad, CA).

Ouchterlony double immunodiffusion was adapted from Bailey (1996). Glass microscope slides were sterilized by spraying 70% alcohol on both sides and wiping with a sterile tissue. They were placed on a levelled table and 4 ml, 1.2% molten agarose dissolved in sodium barbitone solution (pH 8.6) was spread on the glass slide and allowed to set. Circular wells were cut in the gel and these were loaded with desired antigen solutions and antisera. The slides were incubated in a humid chamber for 16 hours to allow immunoprecipitation and then washed in 0.9% saline solution for 48 hours. Enzymatic activity was assayed using both chymotrypsin-like (NAPBNE + FBB) and CES (α -naphthyl acetate + fast red TR) substrates as described below.

Immunofluorescence on the surface of adult worms

Immunofluorescence on adult worms was done as described in Doenhoff *et al* 1988. Briefly, adult worms perfused from infected mice were washed several times in perfusion fluid and fixed in 4% paraformaldehyde in isotonic PBS solution pH 7.4, overnight. Thereafter, the worms were washed thrice in isotonic PBS solution. The fixed worms were incubated in blocking buffer (1% BSA in PBST (PBS + 0.2% Tween 20) for an hour at room temperature and washed in PBST solution.

Primary incubation of the blocked adult worms was done in a 1:40 dilution of a rabbit antiserum raised against the host-derived protease/CES in 1 ml PBST and incubated overnight at 4°C. Worms were washed three times in PBST as above and

incubated in 1 ml 1:80 FITC-labelled secondary goat anti-rabbit IgG (Abcam, Cambridge, UK) in PBST for 30 minutes at room temperature in the dark. The labelled worms were washed 3 times in PBST and examined with the aid of a GX fluorescence microscope (GMXL3201LED) using the 10x objective lens under blue light attached to a GXCAMFLUOMAX camera (GT Vision Ltd www.gxoptical.com).

Mass spectrometric analysis of purified enzymes

Analyses of purified gel bands were carried out using tandem mass spectrometry (Papayannopoulos, 1995, Steen and Mann, 2004). Purified protein was digested by trypsin and fragmented peptides ionised and accelerated in a mass analyser where ion fragments were separated on the basis of mass-to-charge to produce spectra. Data from the resulting mass spectra were searched using the MASCOT software for peptide matching and protein identification. Amino acid sequence searches used the protein basic local alignment search tool (pBLAST) at the National Centre for Biotechnology Information (NCBI) against the non-redundant protein sequences database (nr) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify homologous proteins, while the protein sequence alignment was achieved using ClustalW software (<http://www.ebi.ac.uk/Tools/msa/clustalw/>).

Detection of chymotrypsin-like enzyme activity

Zymography for the detection of chymotrypsin-like enzyme activity in agarose films in RID and SDS-PAGE gels was performed as described by Pearse (1968), adapted as in Darani and Doenhoff (2008), using 5 mg NAPBNE as the chromogenic

substrate and 5 mg FBB as the coupling agent. The substrate mixture was dissolved in 2 ml DMSO and diluted to 40 ml by adding 10 ml, 0.1 M PBS solution, pH 7.4 and 30 ml deionized water. Prior to zymography in SDS-PAGE, the gel was incubated for an hour in 2.5% triton X-100 solution.

Detection of esterase activity

Esterase activity on purified mouse and rat enzyme was assayed using two substrates: β -naphthyl acetate and α -naphthyl acetate. The chromogenic substrate solution for β -naphthyl acetate was adapted from Bahar *et al.* (2012), using fast blue salt as coupling agent dissolved in dimethyl sulfoxide (DMSO) and diluted in 50 mM sodium acetate buffer, pH 7.4. The substrate for α -naphthyl acetate was adapted from Duysen *et al.* (2011) and Otto *et al.* (1981) using fast red TR (BDH chemicals Ltd., Poole, England), as coupling agent, dissolved in DMSO and diluted in 50 mM sodium acetate buffer, pH 7.4.

Inhibition of enzymatic activities

The inhibition of the purified chymotrypsin-like enzyme from the sera of mice and rats was carried out using phenylmethanesulphonyl fluoride (PMSF) (Darani and Doenhoff, 2008). The inhibition of the carboxylesterase activity of the purified enzyme from the sera of mouse and rat using bis-p-nitrophenyl phosphate (BNPP) was adapted from Xie *et al.* (2002). Briefly, the purified mouse and rat enzymes were electrophoresed in replicate 1-D SDS-PAGE gels. After electrophoresis, the gels were first incubated at room temperature in 2.5% triton X-100 solution for an hour (to

allow the enzymes to refold), rinsed thrice in deionised water, followed by a second incubation in 0.1 M PBS solution, pH 7.4 for 10 minutes. Thereafter, the gels were divided into three groups with each of the groups containing three replicates of each of the purified mouse and rat enzymes. The first group was treated for two hours by incubation in 10 mM PMSF dissolved in DMSO and diluted in PBS at 37°C. A second was incubated at 37°C in a solution containing 5 mM BNPP dissolved in PBS for 2 hours, while the third group was incubated only in 1xPBS under the same conditions. The reactions were stopped by washing five times in PBS and the gels immersed in chromogenic substrate mixtures for detection of enzymatic activity. A gel piece was taken from each of the three groups and incubated in three substrate solutions containing the NAPBNE (for the detection of chymotrypsin-like enzyme activity), β -naphthyl acetate and α -naphthyl acetate (both esterase substrates).

RESULTS

Investigating the three fractions arising from the fractionation of NMS for the presence of albumin and the chymotrypsin-like enzyme

Each of the three fractions from albumin-depleted mouse serum (precipitates A and B and the supernatant) was investigated using rabbit anti-MSA and anti-NMS in radial immunodiffusion (RID) for the presence and concentration of albumin and the chymotrypsin-like enzyme (Fig.1).

The presence of the chymotrypsin-like enzyme was indicated by a purplish colour after chromogenic staining using NAPBNE + FBB (Fig. 1b). The results indicated that precipitate 'A' (well 2) had relatively less albumin (Fig. 1a), while retaining more of the enzyme activity than the other fractions and it was therefore used for further purification of the enzyme by means of 1-D SDS-PAGE.

Approximate place for Figure 1

Purification of the chymotrypsin-like enzyme from precipitate A using SDS-PAGE

Further purification of the enzyme from precipitate A was followed by its isolation from three successive SDS-PAGE gels, the band with enzymatic activity being excised from replicate thin strips of the polyacrylamide gel, eluting the enzyme therefrom and re-electrophoresing the product in a further PAGE. The procedure of (i) SDS-PAGE, (ii) elution of enzyme activity from gel strips, (iii) re-electrophoresing in PAGE was repeated two times (Fig. 2). The Coomassie-stained band in lane 5 showing the purified enzyme (blue arrow) was subjected to tandem mass spectrometry (MS) and the results are shown in Table 1. Tandem MS analysis and a

Mascot search of the Swiss-Prot database revealed significant peptide matches with mouse (*Mus musculus*) enzyme carboxylesterase 1C (EST1C_MOUSE) and mouse alpha-1B-glycoprotein (A1BG_MOUSE). The same proteins with identical amino acid sequences are present also in the NCBI database (Mouse CES 1C: GI: 247269929 and NP: 031980.2; Mouse A1BG: GI: 124486702 and NP: 001074536.1).

Approximate place for Figure 2

Approximate place for Table 1

Purification of the chymotrypsin-like enzyme from rat serum

A seemingly analogous chymotrypsin-like enzyme was also found after SDS-PAGE and zymography of normal rat serum (NRS) using the same chromogenic substrate NAPBNE and its coupling agent fast blue B (Darani and Doenhoff, 2008). The purification of the enzyme in NRS was undertaken to help confirm the identity of the mouse-derived enzyme and help in the characterization of the enzyme. Extracts of NRS and NMS sera were loaded into adjacent wells and electrophoresed in 1-D SDS-PAGE. The result of zymography to detect enzymatic activity in NMS and NRS in SDS-PAGE is shown in Fig. 3. The result revealed that the enzyme in NRS has a slightly smaller size in SDS-PAGE compared to that in NMS.

Approximate place for Figure 3

The same method that had been adopted for the purification of the enzyme in NMS was applied to NRS; i.e., albumin depletion followed by SDS-PAGE, elution

from the gel and re-electrophoresing the contents of the eluate. The result is shown in Fig. 4.

Approximate place for Figure 4

The partially purified Coomassie blue-stained band indicated by the arrow in Fig. 4b was subjected to tandem mass spectrometry and derived peptides were searched in MASCOT for protein identification. Significant matches for the peptides identified by MS were given by two protein entries in the SwissProt database, namely rat carboxylesterase 1C (RCES 1C) (Table 2) and rat alpha 1B-glycoprotein (RA1BG) (result not shown). The same proteins with identical peptide sequences are present in the NCBI database (RCES 1C: GI: 2506388 and NP: 10959.3; RA1BG: GI: 25453392 and NP: 071594.2).

The MS result for RCES 1C indicated it was marginally smaller than MCES 1C, as had been indicated by the results of zymography after PAGE (Fig. 3).

Approximate place for Table 2

Investigation of the purified chymotrypsin-like enzyme for esterase activity

Following the indications from MS that the purified host-derived chymotrypsin-like enzyme from mouse and rat plasmas may be carboxylesterases, samples of the enzymes purified from mouse and rat sera were subjected to zymography with two esterase substrates: β -naphthyl acetate and α -naphthyl acetate and NAPBNE as a positive control. The purified enzymes from both mouse and rat were observed to hydrolyse both esterase substrates at the same position in the gel as the activity

against NAPBNE, providing additional evidence for the enzyme in question to be an esterase (Fig. 5).

Approximate place for Figure 5

Inhibition of the chymotrypsin-like enzyme and esterase activities in samples purified from NMS and NRS

The enzyme activities purified respectively from mouse and rat sera and visualized by zymography with the two esterase substrates were further characterized using a carboxylesterase substrate inhibitor: BNPP and the protease inhibitor PMSF. Both inhibitors were observed to have inhibited the ability of both the purified enzymes to hydrolyse the two esterase substrates as well as the substrate of chymotrypsin-like enzymes (Fig. 6b, c, e, f, h, i), as compared to the control groups which were incubated in the absence of any of the two inhibitors (Fig. 6a, d and g).

Approximate place for Figure 6

Enzyme detection in adult worm membrane and purified CES from NMS

The purified CES was investigated in Ouchterlony double immunodiffusion to ascertain its immunological identity with an enzyme in a detergent extract of mouse-derived adult worms and the enzyme in NMS. A rabbit anti-NMS antiserum was loaded in one well while the purified CES and unfractionated WM or NMS were loaded in wells adjacent to each other and opposite the well containing the antiserum. A pattern of identity of precipitin lines formed between the purified CES

enzyme and an antigen in WM and NMS indicated that antibodies in the anti-NMS were immunoprecipitating the same molecule in all 3 antigen solutions (Fig. 7).

Approximate place for Figure 7

Immunofluorescent detection of the host-derived enzyme on adult worms

The presence of the enzyme on the surface of mouse-derived adult worms was investigated. An immunofluorescent test was done by probing the surface of freshly perfused adult worms with rabbit antibodies raised against the chymotrypsin-like enzyme derived from a detergent extract of adult worms (WM), while control worms were probed with rabbit antibodies raised against complete Freund's adjuvant (anti-CFA). Results showed that the surfaces of worms probed with antibodies raised against the chymotrypsin-like enzyme present in a detergent extract of adult worms were immunofluorescent while control worms were not (Fig. 8).

Approximate place for Figure 8

Investigating peptide homology between mouse and rat CES 1C

The homology of the host-derived mouse CES 1C (GI: 247269929, NP: 031980.2) to other mammalian proteins was investigated by a pBlast search of its amino acid sequence on the NCBI database. The search identified rat CES 1C (GI: 2506388, NP: 10959.3) with a score of 913, an E value of 00 and identity of 83%, as the mammalian protein most closely similar to that of mouse CES 1C.

Furthermore, the amino acid sequences of mouse and rat enzymes were investigated for homology with any human protein. The result revealed that both enzymes were homologous with human carboxylesterases, one of the most similar being human CES 1 (monocyte / macrophage serine esterase 1) (GI: 15214585, NP: AAH12418.1) with peptide identity of 65.52%, a score of 759, and an E value of 00 (result not shown). An alignment of the amino acid sequences of the mouse, rat and human CES using the ClustalW software revealed a peptide identity of 83.06% between mouse and rat CES 1C, 65.52% identity between mouse CES 1C and human CES 1 and 71.22% identity between rat CES 1C and human CES 1. MS-derived peptides which matched each of the mouse and rat sequences are highlighted by underlining in Fig. 9.

Approximate place for Figure 9

DISCUSSION

The uptake of host molecules by schistosomes is considered to play a crucial role in immune evasion and enhancement of parasite survival (Dingirard and Yoshino, 2006, McLaren *et al.*, 1975, Skelly, 2004, Smithers *et al.*, 1969). Here, the purification and characterization of an enzyme, apparently with chymotrypsin-like activity, and present in a deoxycholic acid (DOC) detergent extract of *S. mansoni* adult worms (WM) recovered from infected mice, was undertaken to determine its identity and perhaps thus provide insight into its role(s) in the parasite-host relationship.

The enzyme in DOC extracts of the parasite (WM) was observed to be similar antigenically and enzymatically to that in normal mouse serum (NMS) and little or none was present in aqueous extracts of adult worms without DOC (Darani and Doenhoff, 2008). It has here been confirmed (Fig. 7) that the enzyme in mouse blood was identical to that in the detergent extracts of worms and purification from NMS was necessitated because of the limited availability of worm-derived material. The enzyme was previously reported to have a molecular weight of ~70 KDa (Darani and Doenhoff, 2008), and thus quite similar to that of serum albumin. The high concentration of albumin in serum made it difficult to isolate the enzyme and methods to reduce the concentration of the former were therefore employed (Chen *et al.*, 2005, Colantonio *et al.*, 2005).

The method of albumin depletion resulted in fractionating NMS into two precipitates (first and second) after two successive centrifugations and a supernatant containing mainly albumin (Fig. 1). The first precipitate, which had a reduced concentration of albumin but had contained more of the enzyme, was purified further (Fig. 2). Tandem MS-derived peptides showed a significant match for mouse

carboxylesterase 1C (CES 1C) (Table 1), although some MS-derived peptides also matched that of mouse α -1B-glycoprotein (result not shown). The host-derived enzyme was considered most likely to be carboxylesterase as the MS-derived peptides gave a lower match with α -1B-glycoprotein and the latter is not known to exhibit any enzymatic activity. The low molecular weight recorded for the enzyme in MS compared to results from SDS-PAGE could be due to post-translational modification as these enzymes have been reported to be glycosylated (Bernhard *et al.*, 2007, Ghesquiere *et al.*, 2006, Otto *et al.*, 1981).

Further evidence of the identity of the chymotrypsin-like enzyme was obtained from examination of a seemingly analogous enzyme in normal rat serum which in SDS-PAGE and zymography had a stronger staining intensity and a slightly lower molecular weight than the enzyme in mouse serum (Fig. 3). Tandem MS analysis of the partially purified enzyme from rat serum revealed peptides, the sequence of which significantly matched those of rat CES 1C and rat α -1B-glycoprotein (Table 2). Interestingly, the MS-derived peptides matching the aligned amino acid sequences of the mouse and rat enzyme were present in only one or the other sequence, with the exception of two (Fig. 9). A study of the properties and characteristics of the two MS-identified molecules showed similar characteristics / properties between mouse CES 1C and the chymotrypsin-like enzyme. Thus CES's, similarly to the serine protease chymotrypsin, possess a serine residue in their catalytic triad (Fig. 9) (Sato and Hosokawa, 2006, Stoops *et al.*, 1969).

The chromogenic substrate for chymotrypsin-like enzymes (N,N,N acetyl-DL-phenylalanine β naphthyl ester: NAPBNE) has previously been reported to be hydrolysed by rat plasma CES (Choudhury, 1974). The ability of the purified enzymes from both mouse and rat plasma to hydrolyse two esterase substrates (α -

naphthyl acetate and β -naphthyl acetate) (Bahar *et al.*, 2012), is an indication of their esterolytic capability. Moreover, the protein bands with esterolytic activity were observed at the same molecular weight as the band which hydrolysed the chymotrypsin-like substrate (NAPBNE + FBB), a result consistent with the same molecule being active on the three different substrates.

In terms of mode of action of the chymotrypsin-like enzyme and CES, both share very similar active sites in possessing a catalytic triad composed of serine, histidine and either glutamic or aspartic acid (indicated in bold and italicized font in Fig. 9) (Bahar *et al.*, 2012, Brayer *et al.*, 1979, Satoh and Hosokawa, 2006, Stoops *et al.*, 1969). Moreover, the observation that the esterase and chymotrypsin-like activities were both inhibited by PMSF, a chymotrypsin (serine protease) inhibitor and BNPP, a CES inhibitor, indicated similarities of hydrolytic action of the enzymes (Fig. 6). Previous findings on the inhibition of the chymotrypsin-like enzyme and CES in NMS using PMSF and BNPP respectively, further buttress this point (Darani and Doenhoff, 2008, Xie *et al.*, 2002).

The immunoprecipitation of the host-derived enzyme in detergent extracts of the parasite (WM), purified CES and NMS by Ouchterlony double immunodiffusion using a rabbit anti-NMS revealed patterns of immunological identity in both extracts (Fig. 7). This indicates that the purified CES is identical to an enzyme in WM. Moreover, the ability of the immunoprecipitates to hydrolyse substrates of both chymotrypsin-like enzymes (NAPBNE + FBB) and esterases (α -naphthyl acetate) helps substantiate the characterization of the enzyme on worms being host-derived and a CES. Furthermore, immunofluorescence detection on mouse-derived adult worms probed with antibodies specific for the chymotrypsin-like enzyme indicated the host-derived antigen was present on the surface of the worms (Fig. 8). The high

intensity of immunofluorescence observed on adult worms particularly on the female could be an indication of the possible roles of the enzyme for easing the passage of the parasite in host's blood vessels, metabolism and in immune evasion.

Darani and Doenhoff (2008) reported the chymotrypsin-like enzyme to be present with high staining intensity in both mouse and rat plasma, but absent among an array of other mammalian plasmas (hamster; guinea pig, rabbit, bovine or human) that were assayed. The same has been reported for the CES enzyme in the plasmas of mouse and rat among the mammalian plasmas investigated (Bahar *et al.*, 2012, Cerasoli *et al.*, 2000, Li *et al.*, 2005). The reason for CES being present in mouse and rat blood has been explained in terms of their amino acid sequences. Thus, mouse and rat blood contain the secreted form of the CES enzyme. Secretion of CESs from cells in which they are synthesized is normally prevented by the presence of a retention signal tetra-peptide (namely: histidine, X, glutamic acid and a terminal leucine, with X representing any amino acid) at the carboxyl-terminal of the enzyme (Cerasoli *et al.*, 2000). In mice and rats, a disruption in the retention tetra-peptide sequence due to the replacement of the terminal leucine by either threonine or lysine results in the secretion of the CES from the liver into the blood of these animals (Hosokawa, 2008, Satoh and Hosokawa, 2006). Twenty families of carboxylesterase are encoded for in the mouse genome and only one encoded by the ES-1 gene exhibits the disrupted retention signal, meaning that most CES activity in mouse serum is generated in the liver by expression of the ES-1 gene (Duyzen *et al.*, 2011).

A BLAST analysis of sequences in the NCBI database indicated the most similar homologue of mouse CES 1C was rat CES 1C and that human brain CES was the closest human CES to mouse CES 1C (results not shown). However, a

human serine carboxylesterase expressed at high levels in the liver and less in lungs and heart was of particular interest as it has been shown to possess convertase activity in human alveolar lavages and to function as a lung detoxification enzyme (Munger *et al.*, 1991). Similarly to mouse CES 1C, inhibition of the human serine carboxylesterase by PMSF and BNPP has also been reported (Munger *et al.*, 1991). An alignment of amino acid sequences of the mouse, rat and human CES using ClustalW software revealed an identity of 83.06% between mouse and rat CES 1C, 65.52% identity between mouse CES 1C and human CES and 71.22% identity between the rat and human molecules, reflecting homology between all three enzymes (Fig. 9).

A requirement for detergent (DOC) to extract the enzyme into solution suggests it is membrane-bound in the parasite, perhaps on the outer surface. If that is so, several roles could be suggested to explain the presence of CES on the surface membrane. Firstly, mouse CES 1C could be exploited by *S. mansoni* as an immunological disguise for masking surface antigens, thereby preventing the recognition and activation by antigen-presenting cells and activation of the complement system (Furlong *et al.*, 1992).

Alternatively or additionally, the convertase potential of CES 1C (Krishnasamy *et al.*, 1998), could be exploited by *S. mansoni* for inactivating the complement system of the host for the purpose of immune evasion. Another possible role of the CES on the surface of *S. mansoni* could be to neutralize harmful / foreign host molecules which pose a threat to the parasite's survival, specifically those stemming from anti-parasite immune activity. Members of the CES family from the liver microsome in the endoplasmic reticulum are known to hydrolyse and inactivate

foreign substances such as toxins, although their physiological role *in vivo* is as yet unclear (Krishnasamy et al., 1998).

Mouse CES 1C has been shown to be capable of exhibiting convertase activity of lung surfactant subtypes (Genetta *et al.*, 1988, Krishnasamy *et al.*, 1998). *S. mansoni* could thus perhaps 'use' the CES 1C obtained from its mouse host's blood to ease its passage through the host blood vessels as it may help maintain blood vessel stability by inhibiting blood vessel constriction. Mouse blood CES is known to metabolise several pharmaceutical compounds such as temocapril: an angiotensin converting enzyme inhibitor (Bahar *et al.*, 2012, Takai *et al.*, 1997). Angiotensin converting enzyme plays an important physiological role, the outcome of which is a constriction of blood vessels, thereby raising blood pressure. Consistent with this pharmacological role, the enzyme has previously been found by indirect immunofluorescence to be present on the surface of mouse lung-derived schistosomula, but not on the surface of mechanically transformed larvae (Darani and Doenhoff, 2008).

Mammalian CES's are known to be involved in lipid metabolism (Holmes *et al.*, 2010, Meyer *et al.*, 1970, Smith *et al.*, 1970). The metabolic potential of CES could be manipulated by *S. mansoni* in hydrolysing acquired host lipids and lipoproteins, as the parasite cannot synthesize all its needed fatty acids and steroids *ab initio* (Berriman *et al.*, 2009, Brouwers *et al.*, 1997). These are important in the parasite's survival as they serve a crucial role in nutrition, membrane synthesis and maintenance (Dingirard and Yoshino, 2006, Furlong, 1991, Furlong *et al.*, 1992). The high intensity of immunofluorescence observed herein on mouse-derived adult worms particularly on the female could be directly proportional to the above metabolic roles which are crucial for their survival.

Paradoxically, none of the above possible roles of blood-borne CES enable *S. mansoni* to survive well in the rat, a host considered relatively non-permissive for this schistosome species (Cioli *et al.*, 1977), nor apparently is a presence of such an enzyme in the blood of humans a necessity for the *S. mansoni*-permissiveness of that host species. However, the successful characterization of a carboxylesterase seemingly present in extracts of *S. mansoni* is an addition to the list of host-derived molecules acquired by this schistosome species and is the first enzyme to be recorded to have this role. The means by which the molecule adheres to the schistosome remains to be determined. Moreover, the existence of a similar serine carboxylesterase in humans, released by alveolar macrophages, is noteworthy, but investigations to ascertain if it is exploited by the parasite in any of the ways suggested here for mouse CES 1C would of course be difficult.

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ETHICAL AND REGULATORY GUIDELINES

Animals used in this study were maintained according to regulations set out by the UK government and permitted under legislation specified by the Animals (Scientific Procedures) Act 1986.

Table 1: MASCOT search output of tandem MS data from the purified ~85 kDa gel band from mouse serum.

Carboxylesterase 1C (Mus musculus) SwissProt, Mascot Protein Score: 313, Mass: 61017, Coverage: 15%, pI: 4.97		
Peptide match	Peptide Ion Score	Expect
<i>MNEETASLLLR</i>	75	0.0001
<i>EGASEEETNLSK</i>	70	0.00032
<i>APEEILAEK</i>	52	0.019
<i>QKTESELLEISGK</i>	51	0.023
FAPPQPAEPWSFVK	22	18
FWANFAR	22	17
TESELLEISGK	18	56
ISEDCLYLNIYSPADLTK	9	2.8e+02

Peptides with significant scores are shown in italics. Mascot Protein score: The sum of all the peptide ion scores matching a protein; Mass: predicted protein mass in Daltons (Da); Coverage: Percentage of sequence covered by MS-matched peptides; Peptide Ion score: A score assigned to individual matching peptide by Mascot based on the probability of best match; Expect: Frequency of chance of obtaining an equal or higher score for a peptide.

Table 2: MASCOT search output of tandem MS data from the purified ~85 kDa gel band from rat serum. Other details as in the legend to Table 1.

Carboxylesterase 1C (<i>Rattus norvegicus</i>)		
SwissProt, Mascot Protein Score: 182,		
Mass: 60136, pI: 5.51, Coverage: 12%		
Peptide match	Score	Expect
<i>EGASEEETNLSK</i>	69	0.00034
<i>TPEEILTEK</i>	50	0.039
LLADMLSTGK	40	0.31
NPPQTEHTEHT	9	4.1e+02
SFNTVPYIVGFNK	8	4.5e+02
FAPPEPAEPWSFVK	6	7.2e+02

Legends are the same as in table 1

Figure legends

Fig. 1. Radial immunodiffusion of diluted NMS and fractions from albumin-depleted NMS. Immunoprecipitation was carried out with: (a) rabbit anti-whole mouse serum albumin antiserum (anti-MSA) and (b) anti-NMS and stained with chromogenic substrate NAPBNE+FBB. Wells (1) NMS, (2) Precipitate A of albumin-depleted NMS, (3) Precipitate B of albumin-depleted NMS, (4) supernatant from albumin-depleted NMS. (2 μ g of each eluate was loaded into each of the wells).

Fig. 2. Steps towards the purification of the chymotrypsin-like enzyme in normal mouse serum (NMS) using an albumin-depletion method and repeated SDS-PAGEs. (M) Molecular weight markers, (1) NMS, (2 & 3) Precipitate A from albumin-depleted NMS. (4 & 5) Purified enzyme after two successive SDS-PAGEs. Lanes 1, 3 & 5 were stained with Coomassie blue. Lanes 2 & 4 reveal the presence of the enzyme by zymography. Blue arrow shows the gel position of the enzyme.

Fig. 3. Zymography of SDS-PAGE of NRS and NMS to display the enzyme using the chromogenic substrate mixture of NAPBNE + FBB. M: Molecular weight marker (1) NRS, (2) NMS (2 μ g was loaded in each well).

Fig. 4. Purified chymotrypsin-like enzyme in rat serum. (M) Molecular weight marker, (a) zymography of purified rat enzyme, (b) Purified rat enzyme stained in Safe-blue Coomassie stain and investigated in MS.

Fig. 5. SDS-PAGE and zymography investigating the purified enzyme from mouse and rat using two esterase substrates. (a) Stained with chymotrypsin-like enzyme substrate: NAPBNE + FBB, (b) stained with carboxylesterase enzyme substrate: β - naphthyl acetate + FBB, (c) stained with carboxylesterase enzyme substrate: α - naphthyl acetate + fast red TR. (M) Molecular weight markers, (1) purified enzyme from NMS, (2) purified enzyme from NRS.

Fig. 6. Investigating the effect of CES inhibitor bis-p-nitrophenyl phosphate (BNPP) and the protease inhibitor phenylmethanesulphonyl fluoride (PMSF) on purified CES. (a, b & c) was assayed with chymotrypsin-like substrate: NAPBNE + FBB. (d, e & f) was assayed with CES substrate: β -naphthyl acetate + fast blue salt. (g, h & i) was assayed with CES substrate: α -naphthyl acetate + fast red TR. (a, d & g) were controls (b, e & h) were treated with 5 mM BNPP inhibitor. (c, f & i) were treated with 10 mM PMSF inhibitor. (M) Molecular weight marker, (1) Purified enzyme from mouse, (2) Purified enzyme from rat.

Fig. 7. Ouchterlony (Immunodiffusion) and zymography showing that the purified CES from NMS and an antigen in WM are immunoprecipitated similarly by antibodies in a rabbit anti-NMS. Gels 'a' and 'b' (1) rabbit anti-NMS, (2) NMS, (3) WM. Gels 'c' and 'd' (1) rabbit anti-NMS, (2) purified CES, (3) WM. Gels 'a' and 'c' were stained with the chromogenic substrates for chymotrypsin-like enzyme (NAPBNE and FBB), 'b' and 'd' were stained with an esterase substrate (α -naphthyl acetate and fast red TR). All stained gels were photographed over direct light.

Fig. 8. Immunofluorescence probing the surface of mouse-derived *S. mansoni* adult worms. (a) Worm was probed with a rabbit antiserum raised against adult worm-derived mouse chymotrypsin-like enzyme; (b) was probed with a rabbit anti-complete Freund's adjuvant antiserum (anti-CFA).

965 CLUSTAL 2.1 Multiple Sequence Alignments

```

966 Sequence type explicitly set to Protein
967 Sequence format is Pearson
968 Sequence 1: MCES1C      554 aa
969 Sequence 2: RCES1C      549 aa
970 Sequence 3: HCES1       566 aa
971 Sequences (1:2) Aligned. Score: 83.0601
972 Sequences (1:3) Aligned. Score: 65.5235
973 Sequences (2:3) Aligned. Score: 71.2204
974 Guide tree file created: \[clustalw.dnd\]
975
976 Group 1: Sequences:      2      Score: 8261
977 Group 2: Sequences:      3      Score: 8323
978 Alignment Score 7820
979
980 CLUSTAL-Alignment file created \[clustalw.aln\]
981
982 MCES1C      MWLHALVWASLAVCPILGHSLLPPVVDTTQGVLGKYSISLEGFEQPVAVFLGVPFAKPPL
983 RCES1C      MWLCVLVWASLAACPIWGHPPSPVVDTTKGVLGKYSISLEGFTQPVAVFLGVPFAKPPL
984 HCES1       MWLPALVLTATLAASAAWGHPPSPVVDTVHGVLGKFVSLEGFAQPVAIFLGIPFAKPPL
985          ***  .**  *:**. . .  **  *****.:*****.:*****  *****.:*****
986
987 MCES1C      GSLRFAPPQPAEPWSFVKNATSYPPMCSQDAGWAKILSDMFSTEKEILPLKISEDCLYLN
988 RCES1C      GSLRFAPPEPAEPWSFVKNTTTPPMCSQDGVVGKLLADMLSTGKENIPLEFSEDCLYLN
989 HCES1       GPLRFTPPQPAEPWSFVKNATSYPPMCTQDPKAGQLLSELFTRNRKENIPLKISEDCLYLN
990          *.***.***.*****.***.***.***.***.***.***.***.***.***.***.***.***
991
992 MCES1C      IYSPADLTKSSQLPVMVWIHGGGLVIGGASPYNGLALSAHENVVVVTIQYRLGIWGLFST
993 RCES1C      IYSPADLTKNSRLPVMVWIHGGGLIIGGASPYSGLALSAHENVVVVTIQYRLGIWGLFST
994 HCES1       IYTPADLTKKNRLPVMVWIHGGGLMVGAASTYDGLALAAHENVVVVTIQYRLGIWGLFST
995          **.*****. .:*****.***.***.***.***.***.***.***.***.***.***
996
997 MCES1C      GDEHSPGNWAHL DQLAALRWVDNIANFGGNPDSVTIFGE SSGGISVSVLVLSPLGKDLF
998 RCES1C      GDEHSRGNWAHL DQLAALRWVDNIANFGGNPDSVTIFGE SAGGVSVSVLVLSPLAKNLF
999 HCES1       GDEHSRGNWGHLDQVAALRWVDNIASFNGGNPGSVTIFGE SAGGESVSVLVLSPLAKNLF
1000          *****  ***.***.***.***.***.***.***.***.***.***.***.***.***
1001
1002 MCES1C      HRAISESGVVINTNVGKK-NIQAVNEIIATLSQCNDTSSAAMVQCLRQKTESELLEISGK
1003 RCES1C      HRAISESGVVLTTNLDKK-NTQAVAQMIATLSGCNNTSSAAMVQCLRQKTEAEELLETVK
1004 HCES1       HRAISESGVALTSVLVKKGDVKPLAEQIAITAGCKTTTSAVMVHCLRQKTEELLETTLK
1005          *****.: .: : ** : .: : ** : *: *.***.***.***.***.***.***
1006
1007 MCES1C      LVQYNI-----SLSTMIDGVVLPKAPEEILAEKSFNTVPYIVGFNKQEFGWIIIP
1008 RCES1C      LDNT-----SMSTVIDGVVLPKTPEEILTEKSFNTVPYIVGFNKQEFGWIIIP
1009 HCES1       MKFLSLDLQGDPPRESQPLLGTVIDGMLLLKTPEELQAERNFHTVPYIMVGINKQEFGLIIP
1010          : .:*****:* *.***: *: *.***:***.***.***.***.***.***
1011
1012 MCES1C      MMLQNLLPEGKMNEETASLLLRHFHSELNISESMIPAVIEQYLRGVDDPAKKSELILDMF
1013 RCES1C      TMMGNLLSEGRMNEKMASSLLRRFSPNLNISESVIPAIIEKYLRGTDDPAKKKELLDMF
1014 HCES1       MLMSYPLSEGQLDQKTAMSLLWKSYPVLCIAKELIPEATEKYLGTTDDTVKKKDLFLDLI
1015          ::  *.***:***: * ** : . : *::**  *.** *.**..**.:***:
1016
1017 MCES1C      GDIFFGIPAVLLSRSLRDAGVSTYMYEFYRPSFVSDKRPQTVEGDHGDEIFFVFGAPLL
1018 RCES1C      SDVFFGIPAVLMSRSLRDAGAPTYMYEFQYRPSFVSDQRPQTVEGDHGDEIFSVFGTPFL
1019 HCES1       ADVFMGVPSVIVARNHRDAGAPTYMYEFQYRPSFSSDMKPKTVIGDHGDELFSVFGAPFL
1020          .*:***.***:***:*. *****.*****.***** ** :*.** *****.* ***:**
1021
1022 MCES1C      KEGASEEETNLSKMVMKFWANFARNGNPNGEGLPHWPEYDEQEGYLQIGATTQQAQRLKA
1023 RCES1C      KEGASEEETNLSKLVKMFANFARNGNPNGEGLPHWPEYDQKEGYLQIGATTQQAQKLKG
1024 HCES1       KEGASEEEIRLSKMVMKFWANFARNGNPNGEGLPHWPEYNQKEGYLQIGANTQAAQKLKD
1025          *****  .***.*****.*****.*****.: .:*****.*** **.*
1026
1027 MCES1C      EEVAFWTELLAKNPPETDPTTEHTEHK
1028 RCES1C      EEVAFWTELLAKNPPQTEHTEHT---
1029 HCES1       KEVAFWTNLFKAKAVEKPPQTEHIEL
1030          :*****.:***: . . .
1031
1032 MCES1C:0.11318,
1033 RCES1C:0.05621,
1034 HCES1:0.23158);

```

1037 **Fig. 9. An alignment of mouse and rat CES 1C and human CES**

1038 MS derived peptides that match the aligned sequences of mouse and rat CES 1C in
1039 MASCOT are underlined. Residues forming the catalytic triad for CES and
1040 chymotrypsin-like enzymes are shown in bold font and italicised.

1041

1042