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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

62 **KEY FINDINGS**

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

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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 (Dinguirard 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.

128 **MATERIALS AND METHODS**

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

135

136 *Parasite extract and antigens*

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

143 Detergent extracts of *S. mansoni* worms (WM) were prepared by re-
144 suspending freshly perfused, gravity-sedimented worms which had had all visible
145 traces of erythrocytes removed from the suspending fluid, in twice their volume of
146 2% deoxycholic acid (detergent) solution in isotonic saline as previously described
147 by Doenhoff *et al.* (1988). The suspension was gently agitated for 4 hours at room
148 temperature and then centrifuged. The supernatant was removed and stored at -
149 80^0C until used for rabbit immunization or in immunoprecipitation studies.

150

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 4^0C 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 4^0C and the clear supernatant
158 removed and stored at -80^0C 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 -20^0C.

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 4^0C. 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 4^0C. The
191 mixture was centrifuged at 16000 x g for 45 minutes at 4^0C 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 4^0C for 60 minutes. The mixture was centrifuged as above and supernatant

196 containing mainly albumin transferred into sterile micro-tube ‘C’ while retaining the
197 pelleted precipitate. The first and second precipitates were separately reconstituted
198 with 0.1 M PBS, pH 7.4 (weight/volume) and stored at -20^{0}C. The protein
199 concentration in NMS was 23.70 mg/ml while concentrations in solutions of the two
200 precipitates A and B and the supernatant were 5.50, 5.90 and 10.90 mg/ml
201 respectively.

202

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

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

208 Purification of the chymotrypsin-like enzyme in mouse and rat sera was
209 achieved in one-dimensional sodium dodecyl 12% acrylamide gel electrophoresis
210 (Laemmli, 1970) as modified by Studier (1973). 18 ug of each serum (mouse or rat)
211 were loaded into separate gels with broad wells (6.2 cm long) in replicates and
212 electrophoresed. Bands with enzymatic activity were excised from replicate thin
213 strips of the polyacrylamide gel, put into a 1.5 ml Eppendorf container and covered
214 with a minimum volume of elution buffer (0.06 M Tris-HCl, 10% SDS, pH 7.0) (Beyer
215 *et al.*, 2008). The tube was incubated at 37^{0}C for 24 hours, centrifuged at 14,000 x g
216 for 30 minutes at the same temperature and the resultant eluate was removed.
217 Further purification of the enzyme was achieved by re-electrophoresing the eluate in
218 a fresh SDS-PAGE gel. The whole sequence of (i) SDS-PAGE, (ii) elution of enzyme
219 activity from gel strips, (iii) re-electrophoresing in PAGE was repeated three times in

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

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

233

234 *Immunofluorescence on the surface of adult worms*

235 Immunofluorescence on adult worms was done as described in Doenhoff *et al*
236 1988. Briefly, adult worms perfused from infected mice were washed several times in
237 perfusion fluid and fixed in 4% paraformaldehyde in isotonic PBS solution pH 7.4,
238 overnight. Thereafter, the worms were washed thrice in isotonic PBS solution. The
239 fixed worms were incubated in blocking buffer (1% BSA in PBST (PBS + 0.2%
240 Tween 20) for an hour at room temperature and washed in PBST solution.

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

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

249

250 *Mass spectrometric analysis of purified enzymes*

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

262

263 *Detection of chymotrypsin-like enzyme activity*

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

267 substrate and 5 mg FBB as the coupling agent. The substrate mixture was dissolved
268 in 2 ml DMSO and diluted to 40 ml by adding 10 ml, 0.1 M PBS solution, pH 7.4 and
269 30 ml deionized water. Prior to zymography in SDS-PAGE, the gel was incubated for
270 an hour in 2.5% triton X-100 solution.

271

272 *Detection of esterase activity*

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

281

282 *Inhibition of enzymatic activities*

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

290 allow the enzymes to refold), rinsed thrice in deionised water, followed by a second
291 incubation in 0.1 M PBS solution, pH 7.4 for 10 minutes. Thereafter, the gels were
292 divided into three groups with each of the groups containing three replicates of each
293 of the purified mouse and rat enzymes. The first group was treated for two hours by
294 incubation in 10 mM PMSF dissolved in DMSO and diluted in PBS at 37^0C. A
295 second was incubated at 37^0C in a solution containing 5 mM BNPP dissolved in PBS
296 for 2 hours, while the third group was incubated only in 1xPBS under the same
297 conditions. The reactions were stopped by washing five times in PBS and the gels
298 immersed in chromogenic substrate mixtures for detection of enzymatic activity. A
299 gel piece was taken from each of the three groups and incubated in three substrate
300 solutions containing the NAPBNE (for the detection of chymotrypsin-like enzyme
301 activity), β-naphthyl acetate and α-naphthyl acetate (both esterase substrates).
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312 **RESULTS**

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

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

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

324 Approximate place for Figure 1

325

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

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

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

340 Approximate place for Figure 2

341 Approximate place for Table 1

342

343 *Purification of the chymotrypsin-like enzyme from rat serum*

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

353 Approximate place for Figure 3

354

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

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

359 Approximate place for Figure 4

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

368 The MS result for RCES 1C indicated it was marginally smaller than MCES
369 1C, as had been indicated by the results of zymography after PAGE (Fig. 3).

370

371 Approximate place for Table 2

372

373 *Investigation of the purified chymotrypsin-like enzyme for esterase activity*

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

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

382 Approximate place for Figure 5

383

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

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

393 Approximate place for Figure 6

394

395 *Enzyme detection in adult worm membrane and purified CES from NMS*

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

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

404 Approximate place for Figure 7

405

406 *Immunofluorescent detection of the host-derived enzyme on adult worms*

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

415 Approximate place for Figure 8

416

417 *Investigating peptide homology between mouse and rat CES 1C*

418 The homology of the host-derived mouse CES 1C (GI: 247269929, NP:
419 031980.2) to other mammalian proteins was investigated by a pBlast search of its
420 amino acid sequence on the NCBI database. The search identified rat CES 1C (GI:
421 2506388, NP: 10959.3) with a score of 913, an E value of 00 and identity of 83%, as
422 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

448 **DISCUSSION**

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

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

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

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

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

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

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

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

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

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

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

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

548 human serine carboxylesterase expressed at high levels in the liver and less in lungs
549 and heart was of particular interest as it has been shown to possess convertase
550 activity in human alveolar lavages and to function as a lung detoxification enzyme
551 (Munger *et al.*, 1991). Similarly to mouse CES 1C, inhibition of the human serine
552 carboxylesterase by PMSF and BNPP has also been reported (Munger *et al.*, 1991).
553 An alignment of amino acid sequences of the mouse, rat and human CES using
554 ClustalW software revealed an identity of 83.06% between mouse and rat CES 1C,
555 65.52% identity between mouse CES 1C and human CES and 71.22% identity
556 between the rat and human molecules, reflecting homology between all three
557 enzymes (Fig. 9).

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

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

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

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

587 Mammalian CES's are known to be involved in lipid metabolism (Holmes *et*
588 *al.*, 2010, Meyer *et al.*, 1970, Smith *et al.*, 1970). The metabolic potential of CES
589 could be manipulated by *S. mansoni* in hydrolysing acquired host lipids and
590 lipoproteins, as the parasite cannot synthesize all its needed fatty acids and steroids
591 *ab initio* (Berriman *et al.*, 2009, Brouwers *et al.*, 1997). These are important in the
592 parasite's survival as they serve a crucial role in nutrition, membrane synthesis and
593 maintenance (Dinguirard and Yoshino, 2006, Furlong, 1991, Furlong *et al.*, 1992).
594 The high intensity of immunofluorescence observed herein on mouse-derived adult
595 worms particularly on the female could be directly proportional to the above
596 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.

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

831

832 Carboxylesterase 1C (Mus musculus) SwissProt, 833 Mascot Protein Score: 313, Mass: 61017, Coverage: 834 15%, pI: 4.97		
835 Peptide match	Peptide Ion 836 Score	Expect
837 *MNEETASLLLR*	75	0.0001
838 *EGASEEETNLSK*	70	0.00032
839 *APEEILAEK*	52	0.019
840 *QKTESELLEISGK*	51	0.023
841 FAPPQPAEPWSFVK	22	18
842 FWANFAR	22	17
843 TESELLEISGK	18	56
844 845 ISEDCLYLNIYSPADLTK	9	2.8e+02

846

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

853

854 **Table 2**: MASCOT search output of tandem MS data from the purified ~85 kDa gel

855 band from rat serum. Other details as in the legend to Table 1.

856

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

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866 Legends are the same as in table 1

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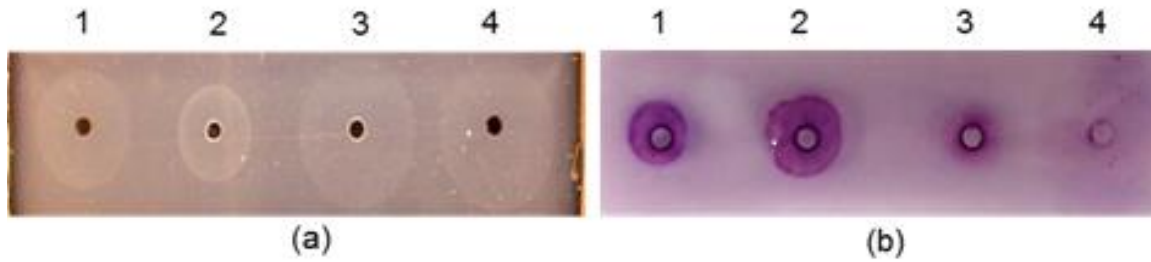
873 **Figure legends**



874

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

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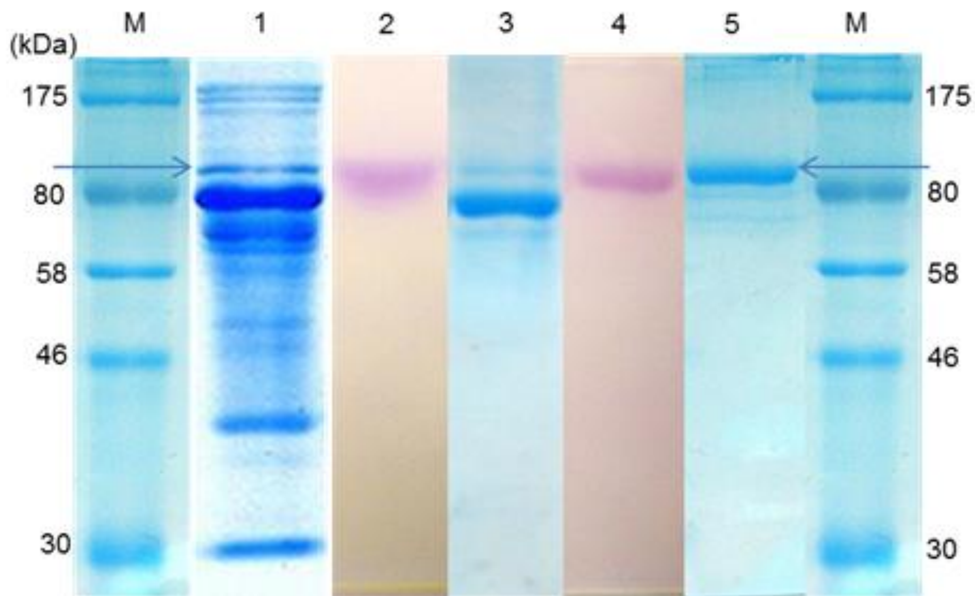

895

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

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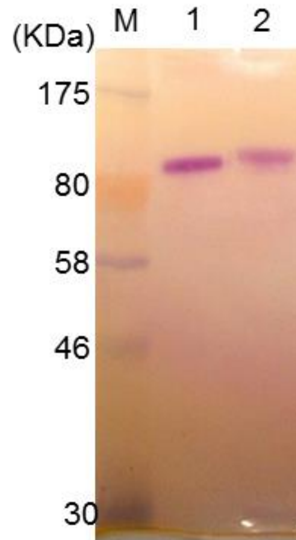

909

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

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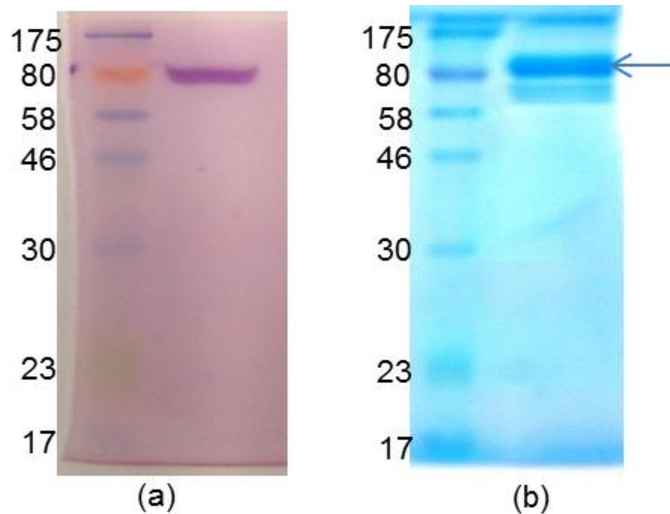

915

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

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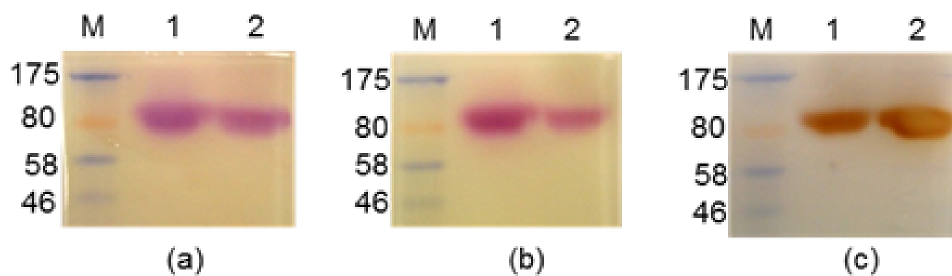

920 **Fig. 5. SDS-PAGE and zymography investigating the purified enzyme from**
921 **mouse and rat using two esterase substrates.** (a) Stained with chymotrypsin-like
922 enzyme substrate: NAPBNE + FBB, (b) stained with carboxylesterase enzyme
923 substrate: β- naphthyl acetate + FBB, (c) stained with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

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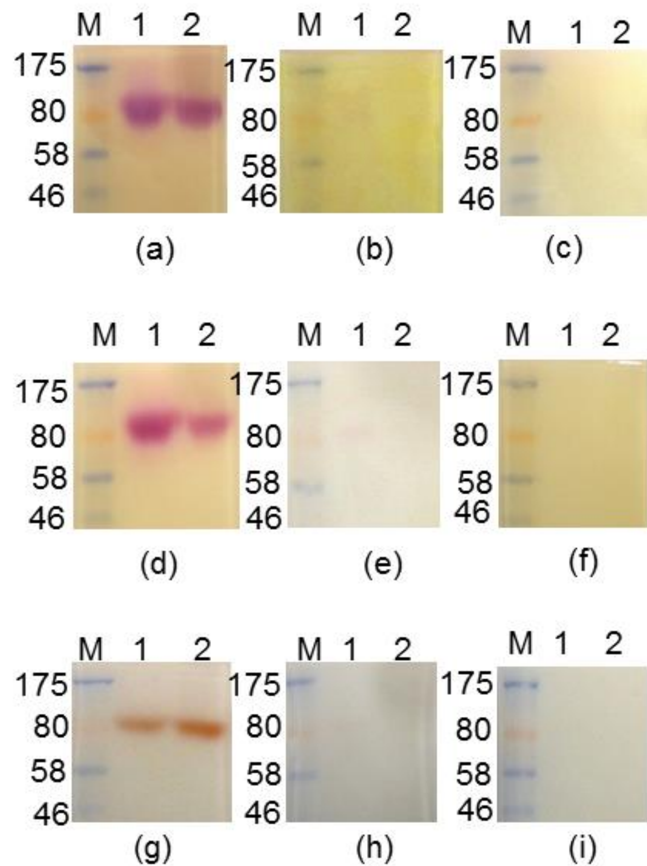

939

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

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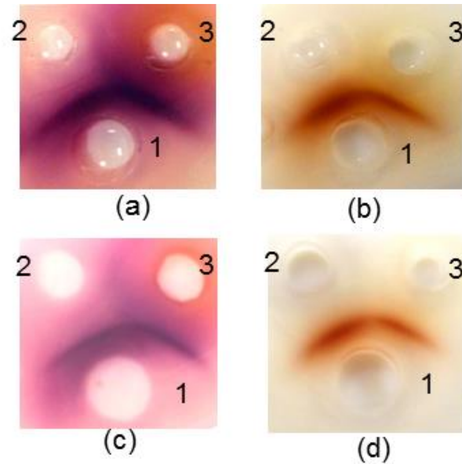
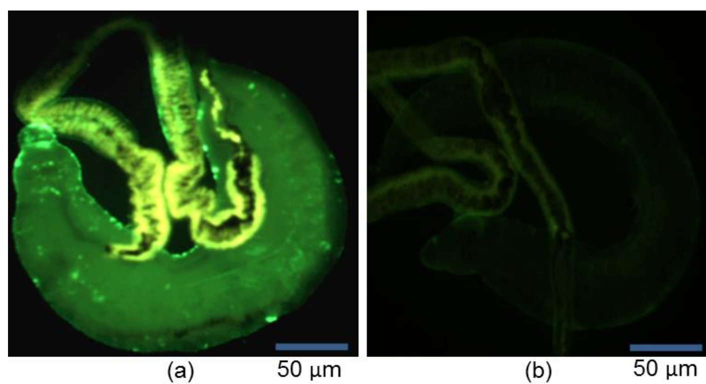


952

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



960

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

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966   Sequence type explicitly set to Protein
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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]
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976   Group 1: Sequences:   2      Score: 8261
977   Group 2: Sequences:   3      Score: 8323
978   Alignment Score 7820
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980   CLUSTAL-Alignment file created [clustalw.aln]
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985               *** .** *:**...  **.  ******.:******::***** ****:***:*******
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988   RCES1C      GSLRFAPPEPAEPWSFVKNTTTYPPMCSQDGVVGKLLADMLSTGKENIPLEFSEDCLYLN
989   HCES1       GPLRFTPPQPAEPWSFVKNATSYPPMCTQDPKAGQLLSELFTNRKENIPLKLSEDCLYLN
990               *.***:**:**********:*:*****:**   .::*:::::. ** :**::********
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993   RCES1C      IYSPADLTKNSRLPVMVWIHGGGLIIGGASPYSGLALSAHENVVVVTIQYRLGIWGLFST
994   HCES1       IYTPADLTKKNRLPVMVWIHGGGLMVGAASTYDGLALAAHENVVVVTIQYRLGIWGFFST
995               **:******..:************::*.**.*.****:******************:***
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1003  RCES1C      HRAISESGVVLTTNLDKK-NTQAVAQMIATLSGCNNTSSAAMVQCLRQKTEAELLELTVK
1004  HCES1       HRAISESGVALTSVLVKKGDVKPLAEQIAITAGCKTTTSAVMVHCLRQKTEEELLETTLK
1005              *********.:.: : ** : :.: : **  : *: *:**.**:******* **** : *
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1007  MCES1C      LVQYNI-----------SLSTMIDGVVLPKAPEEILAEKSFNTVPYIVGFNKQEFGWIIP
1008  RCES1C      LDNT-------------SMSTVIDGVVLPKTPEEILTEKSFNTVPYIVGFNKQEFGWIIP
1009  HCES1       MKFLSLDLQGDPRESQPLLGTVIDGMLLLKTPEELQAERNFHTVPYMVGINKQEFGWLIP
1010              :                 :.*:***::* *:***: :*:.*:****:**:*******:**
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1015               ::   *.**::::: *  ** :  . : *::.:**   *:** *.**..**.:*:**::
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1018  RCES1C      SDVFFGIPAVLMSRSLRDAGAPTYMYEFQYRPSFVSDQRPQTVQGDHGDEIFSVFGTPFL
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1025              ******** .***:*************************:::********.** **:**
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1028  RCES1C      EEVAFWTELLAKNPPQTEHTEHT---
1029  HCES1       KEVAFWTNLFAKKAVEKPPQTEHIEL
1030              :******:*:**:. :.       .
1031
1032  MCES1C:0.11318,
1033  RCES1C:0.05621,
1034  HCES1:0.23158);
1035

1036
```

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

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