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Short communication

Expression and comparative functional characterisation of recombinant acetylcholinesterase from three species of *Schistosoma*

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Parasitic blood flukes of the genus *Schistosoma* inflict a substantial burden of disease on man and livestock. The three principal human parasites, *S. mansoni*, *S. haematobium* and *S. japonicum* are estimated to account for more than 250 million infections in tropical and subtropical regions [1]. Current treatment is reliant on a single effective drug, praziquantel, against which there is evidence of emerging resistance [2–4], and as yet there are no available vaccines. Consequently, there is an accepted need for the development of new therapies to combat this debilitating disease. Prior to the widespread introduction of praziquantel, the drug of choice for the treatment of *S. haematobium* infections was the organophosphate metrifonate, but this was withdrawn from the market because of its toxicity to the host and because it is only marginally effective against *S. mansoni* or *S. japonicum* [5]. The active metabolite of metrifonate, dichlorvos (2,2-dichlorovinyl dimethyl phosphate or DDVP), acts by inhibiting acetylcholinesterase (AChE; EC 3.1.1.7). This enzyme, which is responsible for the hydrolysis of acetylcholine (ACh)—the endogenous ligand for nicotinic acetylcholine receptors (nAChRs)—fulfils a number of crucial physiological roles within the parasite. In the blood stages, *Schistosoma* AChE (*S*AChE) is present both in the muscle and also on the surface tegument. It appears to be derived from a single gene, and the same molecular form fulfils both surface and muscle functions [6,7,8]. Whilst the enzyme located in the muscle has a conventional synaptic function [9], tegumental *S*AChE serves to modulate the interaction between ACh derived from the host and parasite surface nAChRs [10,11], with consequences on the rate of glucose uptake from the host bloodstream [12].

The variation in species sensitivity to the effects of metrifonate has been a major stumbling block in the development of improved alternative anticholinesterase schistosomicides. Although a number of hypotheses have been suggested in order to account for this phenomenon, the issue has not yet been fully resolved. One obvious explanation is that these species distinctions may be a consequence of differences in enzyme kinetics and in an attempt to investigate this possibility, a number of studies have compared values of affinity for ACh or IC_{50} for cholinesterase inhibitors in crude parasite extracts. Unfortunately however, experimental approaches as well as definitions of enzyme activity have varied widely and consequently the results have been inconsistent. In our own experiments, we obtained similar values for enzyme kinetics and inhibitor sensitivities in worm homogenates or tegumental extracts from *S. haematobium*, *S. mansoni* and *S. bovis* [13] but this contradicts the findings of some other groups who have recorded profound differences in sensitivity to inhibitors or substrate between these species (reviewed in [14]). One significant source of variation in these experiments is the presence of additional parasite proteins in the assay medium, which may be competing with substrate or inhibitors or otherwise influencing the measurement of enzyme activity. Whilst procedures have been described for the enrichment of native *S*AChE (either by means of affinity purification or selective extraction from the parasite surface) [15], such methods are inefficient and have failed to produce yields on a scale and purity suitable for application to a definitive biochemical comparison of esterase from metrifonate sensitive and insensitive *Schistosoma*.

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Table 1
Substrate specificity and inhibitor sensitivities of recombinant schistosome AChEs

Enzyme	K_m (μM)		IC_{50} (μM)			
	ATCh	BuTCh	Dichlorvos	BW284c51	Eserine	Propidium
*Sb*AChE	189 ± 7.1	395 ± 33.9	0.59 ± 0.01	2.53 ± 0.10	0.54 ± 0.09	196 ± 6.0
*Sh*AChE	199 ± 9.8[a]	399 ± 2.5[a]	0.58 ± 0.02	1.97 ± 0.07	0.63 ± 0.04	114 ± 6.9
*Sm*AChE	196 ± 13.7	374 ± 3.7	0.56 ± 0.07	2.22 ± 0.19	0.50 ± 0.05	155 ± 4.5

The Michaelis–Menten constant (K_m) was determined using the direct linear plot with substrate concentrations ranging from 0.125 to 8 mM. Inhibitor IC_{50} values (the concentration of inhibitor which results in 50% reduction of ATCh hydrolysis) were derived from the inhibition curves shown in Fig. 1c–f. All values are given as mean ± S.E.M. of three experiments, each performed in triplicate.

[a] Data for *Sh*AChE taken from [7]. Enzyme inhibition by dichlorvos was also defined in terms of inhibitor constants (K_i) by comparing rates of substrate turnover over a range of substrate concentrations in the presence and absence of inhibitor using the EnzPack enzyme kinetics analysis software (Biosoft); these data are discussed in the text.

We recently described the cloning of *S*AChE from *S. haematobium* (*Sh*AChE; GenBank accession number AF279462) and demonstrated that it was possible to express functional recombinant enzyme in *Xenopus laevis* oocytes [7]. With the subsequent identification and cloning of the homologues from *S. mansoni* (*Sm*AChE; AF279461) and *S. bovis* (*Sb*AChE; AF279463) [8], we now have the means to examine the relative functional properties of these enzymes under controlled experimental conditions. Accordingly, for this present report we have expressed recombinant *Sm*AChE and *Sb*AChE. We have compared the biochemical properties of all three recombinant *S*AChEs in terms of enzyme kinetics, substrate specificity and inhibitor profiles in order to address the unresolved questions concerning the possible existence of functional distinctions between these enzymes at the molecular level.

We found that consistent with our previous observations for the expression of *Sh*AChE [7], active *Sm*AChE and *Sb*AChE was secreted from the *Xenopus* oocyte into the incubation medium and since no background esterase activity is evident, assays evaluating recombinant enzyme function could be applied directly to this medium. Secreted enzyme activity was evident within 24 h of cRNA injection, reached a peak after 72 h but persisted for the 6-day collection period. All three *S*AChEs showed functional characteristics consistent with acetylcholinesterases obtained from other species, including a marked specificity for the hydrolysis of ATCh over BuTCh (Fig. 1a). It was interesting to discover that the K_m values for ATCh and BuTCh were similar for all three recombinant enzymes (Table 1), since previous species-comparative data obtained using crude schistosome homogenates have provided conflicting reports for K_m in these three species. For example, whilst one study [18] found that the K_m for ACh differs markedly between *S. haematobium*, *S. bovis* and *S. mansoni* (here, the K_m for female *S. mansoni* was recorded as being 33 times higher than for female *S. haematobium*), others reported that the values were similar for *S. haematobium* and *S. mansoni* extracts [19,20].

Excess substrate inhibition, a feature associated with binding of substrate to the peripheral anionic site (PAS) was evident in all three *S*AChEs at ATCh concentrations higher than 10 mM (Fig. 1b), and this is similar to the sensitivity to excess substrate reported previously for native AChE extracted from *S. mansoni* [21]. These concentrations are relatively high compared with those required to produce inhibition in many other AChEs; for instance with recombinant human esterase, substrate inhibition by ATCh occurs at 1 mM [22]. These differences appear to be due to the substitution of specific residues responsible for substrate binding at the PAS and the relaying of the resulting conformational changes to the enzyme's active centre, as discussed previously for *Sh*AChE [7].

The effect of various inhibitors on the recombinant *S*AChEs was evaluated (Table 1; Fig. 1c–f). Significantly, we found that recombinant AChE from all three species were equally sensitive to dichlorvos (Fig. 1c) and this is consistent with the findings of some earlier reports on the comparative effects of this inhibitor on AChE activity present within *S. haematobium* and *S. mansoni* extracts [20,23]. Dichlorvos inhibition was determined to be predominantly competitive, and inhibition constants (K_i) of 230 ± 15, 204 ± 14

Fig. 1. Panels (a) and (b): Substrate specificity and the effects of substrate concentration on recombinant schistosome AChE, represented by *Sh*AChE. (a) Michaelis–Menten curves comparing the rates of hydrolysis of ATCh (filled circle) and BuTCh (open circle). (b) Excess substrate inhibition was observed with ATCh concentrations greater than 10 mM. Assays were performed in triplicate and error bars represent standard deviation. Panels (c–f) inhibition of *Sb*AChE (open diamond and broken line), *Sh*AChE (open square and solid line) and *Sm*AChE (filled circle and dotted line) by (c) dichlorvos, (d) BW284c51, (e) eserine or (f) propidium. Samples were pre-incubated with inhibitor at the concentrations indicated for 20 min prior to addition of ATCh at a final concentration of 1 mM. Error bars indicate S.E.M. of three experiments, each performed in triplicate.The recombinant acetylcholinesterases were expressed in stages IV and V *Xenopus laevis* oocytes from cRNA encoding the complete coding regions for *Sh*AChE, *Sm*AChE or *Sb*AChE [8]. The methodology for generating oocyte expression *S*AChE constructs using the pSP64t vector [16] has been described previously [7]. Following cRNA injection, batches of 20 oocytes were incubated at 19 °C in 1 ml modified Barth's medium supplemented with 1 mg/ml bovine serum albumin. The incubation medium was collected and replaced every 24 h for up to 6 days post-injection. Recombinant acetylcholinesterase secreted into this incubation medium was assayed spectrophotometrically according to the principals of Ellman [17], using a 96-well microtitre plate format plate format as detailed in [7]. All readings were corrected for the spontaneous non-specific hydrolysis of substrate.