

## A COMPARATIVE STUDY OF ACETYLCHOLINESTERASE ACTIVITY IN BOVINE (*S. Cervi*) AND HUMAN (*B. malayi*, *W. bancrofti*) FILARIA

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### ABSTRACT

*Setaria cervi*, a bovine filarial parasite, contains a significant amount of acetylcholinesterase (AChE) activity with microfilaria having five to ten times more AChE activity than female and male adult worms, respectively. Because AChE shows substrate specificity and hydrolyzes acetylthiocholine but not butyrylthiocholine, this parasitic enzyme is likely a true acetylcholinesterase. The latter also resembles an AChE enzyme in the human filarial parasite *B. malayi* which hydrolyzes acetylthiocholine iodide three times faster than butyrylthiocholine iodide. The *S. cervi* AChE, like its counterpart, also exhibit inhibition with eserine, a specific inhibitor of this enzyme. Subcellular localization of AChE in adult female worms shows enzyme activity both in the mitochondrial and post-mitochondrial fraction. However, enzyme activity in the soluble fraction is twenty-seven times greater than in the mitochondrial fraction.

*In vitro* cultivation of adult female worms and the microfilarial stage of *S. cervi*, shows AChE activity in the culture medium. The released AChE activity and progressive increase with time suggests active secretion of this enzyme by the parasite. Release of AChE is inhibited by diethylcarbamazine (DEC) and two other antifilarial drugs (centparazine and 72/70) but not levamisole.

These findings suggest that the release of AChE is unaffected but its activity is inhibited by the antifilarials. The similarity of *S. cervi* AChE with *B. malayi* AChE suggests that inhibition of this bovine parasitic enzyme may be used to study the efficacy of antifilarial drugs for use in human filariasis.

Acetylcholinesterase (AChE) is present in filarial parasites. This enzyme is of interest as a possible target of antihelminthic drugs. Of perhaps greater importance, in view of the unique association of host and parasite, is the *in vitro* secretion of these enzymes. Acetylcholinesterase's are secreted by a number of nematodes during *in vitro* cultivation. The intestinal parasite *Trichostrongylus* releases AChE when maintained *in vitro* and antibodies to worm AChE (classification 3.1.1.7) has also been detected in the sera of rats infected with *N. brasiliensis*. The nodular worm of cattle *esophagostomum radiatum* also releases AChE freely when incubated *in vitro* (2) and cattle exposed to infection with this parasite become highly resistant to infection (3) suggesting the development of acquired immunity.

AChE secretion constitutes one level of immunomodulation by parasites, and this enzyme may be an important target of the host immune response. When secreted by gastrointestinal nematodes, AChE's are recognized as specific antigens (4-6).

In view of the practical difficulties in procuring human strain filaria (*W. bancrofti* or *B. malayi*), the bovine filarial parasite *S. cervi* which resembles the human species in having nocturnal periodicity was chosen as an experimental counterpart. Because large quantities of microfilariae free from embryo/egg were obtainable by microdissection of the gravid females of *S. cervi*, we examined the localization and characterization of AChE in both the adult worm and microfilariae of this parasite. The effect of diethylcarbamazine (DEC) on secretion of AChE was also compared to circulating AChE as reported in *B. malayi* and *W. bancrofti*.

#### MATERIALS AND METHODS

Adult female worms of *S. cervi* were collected as described previously (5), and washed thoroughly with Ringer solution and stored at  $-10^{\circ}\text{C}$ . The microfilariae of *S. cervi* were obtained by dissecting gravid females as described by Singhal et al (7) incubating the distal portion of the uterus (1cm) in Ringer solution at  $37^{\circ}\text{C}$  for 2-3 hrs. Microfilariae released into the medium were separated from embryo (5 to 10%) by gel filtration through a sephadex G-25 column and collected by mild centrifugation. The intact microfilariae remained alive and active for 3 days at  $4^{\circ}\text{C}$ . The wet weight of one million microfilariae was  $\sim 36.6\text{mg}$ . Microfilariae were sonicated in KCl (150mM; 1:10w/v) using MSE 150 watt ultrasonic disintegrator Mk<sub>2</sub> at 20Kc in cold. The resultant homogenate was centrifuged at 800g and the supernatant was used for enzymatic studies. AChE was assayed according to the procedure of Ellman et al (8). The assay mixture contained phosphate buffer pH 8.0 (260mM), DTNB, pH 7.0 (1mM) and enzyme protein (100-200 $\mu\text{g}$ ). After adding the substrate (acetylthiocholine iodide 1.5mM), the change in absorption/min was recorded at 412nm, in a spectronic 1001 spectrophotometer. A unit of AChE was expressed as mol of acetylthiocholine iodide hydrolyzed/min/mg of tissue. Protein was measured according to the method of

Lowry et al (9). *In vitro* effect of antifilarials on the AChE enzyme of microfilariae and adult worms was studied aerobically incubating motile parasite in KRB buffer (pH 7.4) for two hr at  $37^{\circ}\text{C}$  with DEC, centparazine and compound 72/70 (0.1-1.0mM) in the presence of glucose (50 $\mu\text{g}/\text{ml}$ ). After incubation, microfilariae and adult worms were removed from the medium, washed three times with drug free medium, homogenized and sonicated for 5 min in ice cold KCl (150mM 1:10w/v). The sonicate was centrifuged at 2000g and used for AChE enzymatic studies as described.

#### RESULTS

Table 1 shows AChE levels in microfilariae and adult worms of *S. cervi* compared with previous report in *B. malayi*. Microfilariae contained five to ten times more AChE activity in the female than in the male worm. The *S. cervi* enzyme hydrolyzed the substrate acetylthiocholine iodide but not butyl thiocholine.

Table 2 shows localization of AChE in adult female worms of *S. cervi*. AChE was detected in both the mitochondrial and post-mitochondrial fraction. The specific activity of post mitochondrial fraction [particulate free (10500xg) supernatant] was 29 times greater than the mitochondrial fraction.

Table 3 shows the secretion of AChE by microfilariae and adult (female) stage of *S. cervi* during *in vitro* incubation. Microfilariae released 20 times more AChE than the adult worm on the basis of per gram wet weight.

Table 4 shows the release of proteins (presumably containing excretory-secretory antigens) and AChE during different time intervals. Protein release exhibited a regular increase up to 6 hr of incubation. However, AChE activity in the incubate was constant after 4 hr of *in vitro* incubation.

Table 5 shows the effect of antifilarials on the activity of AChE compared with circulating AChE of human filaria (*W. bancrofti*) and total folin positive protein released from adult females of *S. cervi* during *in vitro* incubation for 2 hrs in KRB

**TABLE 1**  
AChE Levels (Mean±SE; n=10) in Adult Female Worms  
and Microfilariae of *S. cervi* and *B. malayi*

Host	<i>S. cervi</i> <sup>a</sup> AChE activity in homogenate	<i>B. malayi</i> <sup>b</sup> AChE activity in extract
Adult worm	0.923±0.007	86±0.009
Microfilariae	12.600±0.005	321±0.060

a—n moles of acetylthiocholine iodide hydrolyzed/mg/min at 37°C.  
b—Values according to Rathaur et al (3).

**TABLE 2**  
Localization of *S. cervi*  
Acetylcholinesterase (AChE)  
(mean±SE; n=10)

Tissues	AChE activity*
Homogenate	2.166±4.3
Mitochondrial Fraction	0.108±3.1
Post-Mitochondrial Fraction [Particulate Free (105,00xg) supernatant]	8.166±2.2

\*μ moles of acetylcholinesterase hydrolyzed/mg protein/min at 37°C.

**TABLE 3**  
AChE Secretation (mean) by the  
Adult Female Worm  
and Microfilariae (*S. cervi*)

Host	Rate of Release of AChE*
Adult worm	0.65
Microfilariae	17.40

\*μ moles of acetylcholinesterase hydrolyzed/hr/wet wt of tissue at 37°C.

(pH 7.4) medium. Protein release remained unaffected while activity of AChE was lowered during 2 hr contact with antifilarials. At 100μm concentration centparazine was most effective in inhibiting AChE by 75% followed by DEC (55%) and 72/70 (35%). Levamisole failed to alter AChE activity even at the 100μm concentration. These drug treated worms released AChE at normal levels when further incubated in drug free incubation medium.

*S. cervi* AChE showed optimal activity around pH 8.0 (Fig. 1). The effect of varying concentration of acetylthiocholine iodide on

AChE activity is seen in Fig. 2. Km value from lineweaver-Burk plot is  $7.326 \times 10^{-5}$  M.

Table 6 shows inhibition of AChE from *S. cervi* by eserine sulphate.

#### DISCUSSION

Nematode AChE constitutes a particularly well defined group of parasite excretory-secretory products and may be implicated as playing an important role in the host-parasite relationship (3). The human filarial nematode *B. malayi*, both in the adult female worm and microfilarial

**TABLE 4**  
**Release of Acetylcholinesterase by Adult Female Worm (*S. cervi*)**  
**at Different Time Intervals**

Incubation Time (hr)	Total Protein (in the medium) ( $\mu\text{g}$ )	Activity* ( $\mu$ moles/ml/min)
2	114	0.061 $\pm$ 0.002
4	260	0.130 $\pm$ 0.020
6	386	0.130 $\pm$ 0.020

Motile worms (200mg) were incubated at 10ml KRB buffer for different time intervals at 37°C. After desired time period worms were removed and KRB buffer was used for measuring the activity.

\*Activity values are mean $\pm$ SE of 10 different determinations.

**TABLE 5**  
**Effect of Antifilarials on the Activity of Released**  
**Acetylcholinesterase (*S. cervi*) Compared with *W. bancrofti***

Agent	Final Concentration (mM)	% Activity <sup>a</sup> remaining	% Activity <sup>b</sup> remaining
None	*	100.00	100.00
Centperazine	0.1	25.00	ND
	1.0	15.00	ND
Diethylcarbamizine	0.1	45.00	25.00
	1.0	33.00	15.00
Levamisole	0.1	100.00	40.00
	1.0	85.00	30.00

a—equal number of active worms were incubated in Krebs Ringer (pH 7.4) at 37°C with different antifilarial agents. After 2 hr activity was measured as described (see Materials and Methods).  
b—As reported by Misra et al in *W. bancrofti* (16).  
ND—Not Determined.

stage secretes *in vitro* some form of AChE (3). The presence of AChE has been demonstrated histochemically in *Dipetalonema viteae* (10) while in *W. bancrofti* microfilariae AChE activity has been localized to

a variety of non-neural tissue (11).

AChE, usually considered to have a prime role in neuromuscular transmission, is present in significant amounts in both microfilariae and adult worms of *S. cervi*

**TABLE 6**  
**In Vitro Effect of Eserine Sulphate**  
**on the Activity (% Control)**  
**of AChE from *S. cervi***

Concentration	% Inhibition
1 $\mu$ mol	85
10 $\mu$ mol	90
100 $\mu$ mol	95

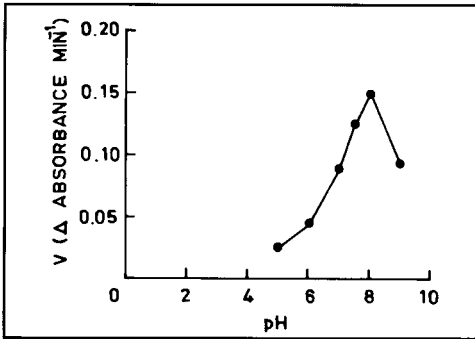


Fig. 1. Effect of pH on AChE activity in *S. cervi*.

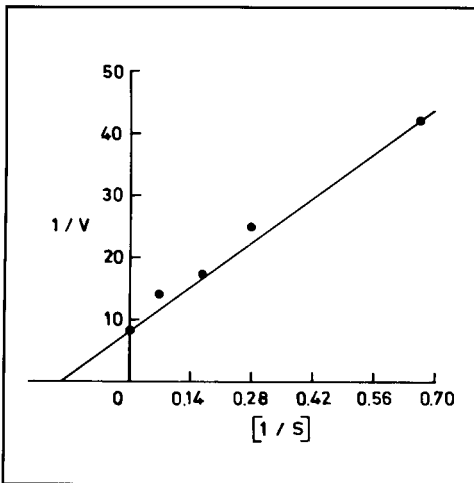


Fig. 2. Lineweaver Burk's double reciprocal plot for calculating the  $K_m$  values. The concentration of DTNB is constant (0.22mM). Enzyme concentration is 3.3 $\mu$ g/ml; other conditions are standard as in enzyme assay.

although the former contain five times more AChE than the latter. *B. malayi* microfilariae similarly demonstrate 3.7 times more activity than the adult female worm (3) whereas *H. contortus* larvae and fourth stage larvae of *T. colubriformis* (5) and *O. radiatum* (4) also display higher levels of AChE as compared to adult counterparts. In contrast, Sanderson and Ogilvie (12) observed a regular increase in AChE activity of *N. brasiliensis* during parasite development.

Our results show that the maximum specific activity of AChE is located in the particulate free (105,000g) supernatant fraction. *S. cervi* AChE is a soluble enzyme. The distribution of this enzyme in the sub-cellular fraction of homogenate of various nematodes is important because association of the bulk of AChE with the particulate fraction may indicate that this enzyme is not readily secreted. High levels of AChE have been detected in the secretory gland of some parasitic nematodes including the human hookworm. Thus, with culture of *Necator americanus* and the rat hookworm, *N. brasiliensis* AChE may be synthesized, secreted and collected in culture medium (13). *S. cervi* microfilariae releases 13 times more enzyme than the adult female worm. When adult worms are incubated for several hrs, activity increases (up to 4 hrs) suggesting that secretion of AChE is an active process.

Human microfilariae (*B. malayi*) secrete AChE 1.4 times more than *B. malayi* adult female worms. The cholinesterase activity of *H. contortus* homogenate fraction recorded a maximum secretion at pH 8.3 while in *S. cervi* the soluble fraction was maximal at a pH of 8.0. The *W. bancrofti* circulating AChE also had an optimal pH of 8.0.

*S. cervi* AChE hydrolyzes the substrate acetylthiocholine iodide but not butyrylthiocholine iodide. *B. malayi* enzyme similarly shows substrate specificity with acetylthiocholine iodide (3). Substantial hydrolysis of acetylcholine iodide is a common characteristic of a true AChE. *S. cervi* AChE does not show excess substrate inhibition and exists in soluble form,

features resembling a non-specific cytosolic thiocholinesterase. Thus, the cholinesterase from *S. cervi* displays properties which to some extent resemble a mammalian acetylcholinesterase. Nevertheless, a definitive classification of *S. cervi* AChE is difficult because not all of its properties conform to a classical acetylcholinesterase. This disparity is in accord with other nematode parasites (14).

When the active worms of *S. cervi* are incubated in KRB medium with antifilarial agents (0.1M concentration), the secreted AChE is inhibited by DEC, centaprazine and 72/70 but not levamisole. These active drugs, however, do not show an effect on tissue bound AChE. The findings suggest that AChE is inhibited only when the antifilarial drug comes directly in contact. Centaprazine at 0.1mM is the most effective against secreted AChE followed by DEC and 72/70. When the worms are removed from the culture medium with the antihelminthic drug, they secrete normal amounts of AChE suggesting that enzyme secretion is not inhibited by the antifilarial. Haloxan (another antihelminthic drug) also inhibits AChE activity of *H. contortus* (15) and *A. lumbricoides* during *in vitro* incubation with this agent.

Antifilarials may inhibit secretion of AChE, a phenomenon which may relate to immunoresponsiveness of the host. Recently, Misra et al (16) have suggested that AChE from *W. bancrofti* infected serum shows significant inhibition with DEC both *in vitro* and *in vivo*.

How release of AChE benefits the parasite is unclear. Host immunity is induced by live worms and secretory antigens are considered a prime stimulus for host immunoresponsiveness (17). Edwards et al (18) have suggested that the secretion of parasite enzymes prevent worm dislodgement from a preferred habitat by inhibiting host-parasite reaction in the host site. In light of the resemblance of *S. cervi* AChE reactivity to its human counterpart (*B. malayi* and *W. bancrofti*) assay of AChE in this bovine filaria may be useful to study the effect of putative antifilarials.

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