Acetylcholinesterase Secretion by *Brugia pahangi in vitro*

Masaaki SHIMADA[1], Eisaku KIMURA[2], Makoto SAKAMOTO[1], Yasunori FUJIMAKI[1], and Yoshiki AOKI[1]

[1]*Department of Parasitology, Institute of Tropoical Medicine, Nagasaki University, 12−4 Sakamoto-machi, Nagasaki 852, Japan*
[2]*Department of Parasitology, Aichi Medical University, Yazako, Nagakute, Aichi-gun, 480−11, Japan*

Abstract: The experiment was designed to demonstrate acetylcholinesterase (AChE) secretion by *Brugia pahangi in vitro.* Seventy five *B. pahangi* adult female worms were cultured in 2 ml of Eagle's minimal essential medium (MEM), and the culture fluid was measured for AChE activity by the colorimetric method of Ellman *et al.* (1961). The enzymic activity increased by the incubation time during the first 24 hours of culture. In an experiment where 25−150 female worms were cultured for 24 hours, higher enzymic activity was detected in the culture fluid where the larger number of worms were cultured. Since AChE activity was not detected in the culture fluid where 100,000 microfilariae were cultured, the results showed that the AChE was secreted by *B. pahangi* adult female worms. The adult male worms did not have enzyme secreting potential. The secreted AChE was identified as a single protein by disc electrophoresis and possessed the characteristics of the true AChE for its substrate specificity and inhibition by eserine.

Key words: Acetylcholinesterase, Secretion, *in vitro, Brugia pahangi*

INTRODUCTION

The morphological features of nematode amphidial glands are those of active secretory cells. Some species of nematode synthesize, store and release their gland secretions. AChE is one of the biologically active substances secreted from amphidial glands, although its role in host-parasite relationship is still unknown (McLaren, 1976). The amphidial glands have been identified in some species of filarial worms (McLaren, 1976). Therefore, it is highly probable that filarial worms secrete AChE *in situ* in the host. The experiments to demonstrate the *in vitro* secretion of AChE by filarial worms, however, provided inconsistent results; *Dipetalonema vitea* was proved to be negative (cited from McLaren, 1976) and *B. malayi* was proved to secrete AChE (Rathaur *et al.*, 1987).

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Therefore, we were encouraged to examine whether AChE is actively secreted *in vitro* by. *B. pahangi,* a lymphatic-dwelling filarial worm which morphologically and physiologically resembles *B. malayi.*

MATERIALS AND METHODS

Collection and Sterilization of Parasites

Adult worms, microfilariae and 4th−stage larvae of *B. pahangi* were recovered from the peritoneal cavity of jirds which had previously been infected by infective *B. pahangi* larvae. Microfilariae were further purified by agar−enclosing method (Nogami *et al.,* 1982). The parasites were then washed 3 times with sterile Hanks' Balanced Salt Solution (HBSS) containing streptomycin (100 μg/ml) and penicilin (100 U/ml).

Secretion of AChE by B. pahangi

Adult worms , microfilariae and the 4th-stage larvae were cultured in 2 ml of the medium containing antibiotics at the concentration given above at 37℃ in 5% CO_2 atmosphere. The culture medium used in our experiments were Eagle's MEM, HBSS and Dulbecco's PBS (−).

Determination of AChE Activity

The AChE activity of the culture fluids was determined by the colorimetric method of Ellman *et al.* (1961) using acetylthiocholine iodide as substrate. Butyrylthiocholine iodide and propionylthiocholine iodide were substituted as substrates in the tests to determine the substrate specificity.

Disc Electrophoresis

Disc electrophoresis was carried out to examine whether secretory AChE shows multiple molecular forms. The culture fluid was concentrated on Amicon Diaflo ultrafiltration membrane, PM−10. Disc electrophoresis was performed by the method of Skangiel-Kramska & Niemierko (1975) on 7.5% polyacrylamide gel at 4℃ for 2 hours at 2.5 mA per tube. AChE from electric eel (Boehringer Mannheim Yamanouchi, Tokyo, Japan) was electrophoresed similtaneously.

Staining of Gels

AChE activity was demonstrated on gels by the procedure of Tsuji (1974).

RESULTS

Secretion of AChE by B. pahangi adult worms in vitro

The levels of AChE activity found in 2 ml of culture fluid in which 75 adult female worms were maintained for 2 − 24 hours are given in Table 1. The results show that the longer the incubation time, the higher the AChE activity of the fluid. The duration of AChE release *in vitro* and the effect of crowding of worms were tested by incubation of each sex of adult worms. Twenty-five to 150 female worms or 100−300 male worms were cultured in 2 ml of Eagle's MEM. The medium was replaced daily and amount of enzyme released each day by the worms was measured, and the results were shown in Table 2.

Table 1. Acetylcholinesterase release by *B. pahangi* adult female worms *in vitro* as a function of time.

Incubation hours	Enzymic activity of culture fluid* U/ml ($\times 10^{-5}$)
2	6.1
4	9.2
8	15.3
12	27.5
24	45.9

*75 adult female worms were cultured in 2 ml of Eagle's MEM at 37℃ in 5% CO_2 atmosphere. Worms were removed at varying times and culture fluid was measured for AChE activity.

Table 2. Duration of acetylcholinesterase release by adult worms *in vitro* and effect of crowding of worms on enzyme release

Day of culture	Number and sex of adult worms in 2 ml of Eagle's MEM							
	Female					Male		
	25	50	75	100	150	100	280	300
1	2.3	4.1	5.1	6.0	7.8	–	–	–
2	0.9	3.2	3.7	4.1	3.7	–	–	–
3	1.8	2.3	1.8	2.3	0.9	–	–	–
4	1.3	2.0	1.3	1.6	0.7			
5	–	2.3	2.3	2.3	–			
7	–	2.0	2.6	1.6	–			
9	–	2.0	2.0	1.6	–			
11	–	1.3	2.0	2.0	–			
13	–	–	2.0	1.3	–			
15	–	–	–	–	–			

For the first 24 hours, the highest activity was recorded in the medium where the largest number of adult female worms (150 worms/2 ml) were cultured. However, in this sample AChE activity declined rapidly and on the 5th day enzymic activity was not detectable. When 50−100 female worms were cultured, the release of AChE continued until day 11−13 of culture. In contrast, no secretory AChE was detected in the fluid where adult male worms were cultured.

AChE Secretion in Other Culture Medium

The adult worms (female 75−100; male 250−380) were cultured in 2 ml of HBSS or Dulbecco's PBS (−) for several days at 37℃ in 5% CO_2 atmosphere. Although the worms were motile in these medium, AChE activity was not detected in either medium (data not shown).

Secretion of AChE by Microfilariae and the 4th Stage Larvae

When microfilariae (100,000 microfilariae/2 ml) or 20 day old larvae (75−270 larvae/2 ml) were cultured in Eagle's MEM for 24 hours, the culture fluid did not show AChE activity (data not shown).

Substrate Specificity of Secretory AChE

The substrate specificity was determined by using butyrylthiocholine iodide and propionylthiocholine iodide as substrate in comparison with an identical concentration of acetylthiocholine iodide. The enzyme released by *B. pahangi* adult female worms hydrolyzed butyrylthiocholine and propionylthiocholine less rapidly than acetylthiocholine (Table 3).

Effect of Eserine on Enzymic Activity of Secretory AChE

The inhibitory effect of eserine sulfate, a specific inhibitor of AChE, on the enzymic activity was tested, and the results were shown in Table 4. At the concentration of 10^{-7} M, eserine inhibited over 70% of the enzymic activity.

Examination of Multiple Forms of Emzyme by Disc Electrophoresis

Densitometric recording of secretory AChE by *B. pahangi* female worms was shown in Fig. 1. A single band was stained on the gel. Although the molecular weight was not determined, the relative mobility of AChE secreted by *B. pahangi* female worms was close to that of AChE from electric eel, the molecular weight of which was reported 230,000 (data not shown).

Table 3. Substrate specificity of secretroy acetylcholinesterase

Substrate	Enzymic activity (U/ml)
Acetylthiocholine Iodide	5.50×10^{-4}
Butyrylthiocholine Iodide	1.37×10^{-4}
Propionylthiocholine Iodide	1.53×10^{-4}

Table 4. Percentage inhibition of secretory acetylcholinesterase activity caused by eserine

Concentration of eserine (M)	% inhibition
10^{-8}	32*
10^{-7}	73
10^{-6}	95
10^{-5}	100

*mean of 2 experiments

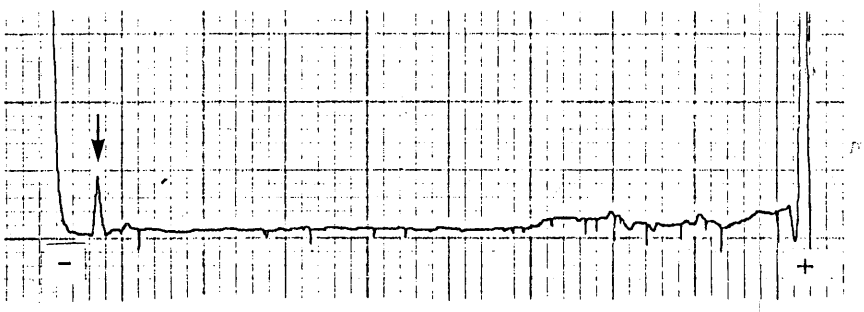

Fig. 1. Densitometric recording of secretory AChE from *B. pahangi* adult female worms. Culture fluid was separated by electrophoresis on 7.5% polyacrylamide gel. AChE activity (arrow) was detected according to Tsuji (1974) and measured on densitometer.

Discussion

The results obtained in our present study provided evidence that AChE is actively secreted by *B. pahangi* adult female worms, especially during the first 24 hours of culture. For adult male worms, we cultured as many as 300 worms in 2ml of Eagle's MEM for 24 hours to examine AChE activity in the medium, because the adult male worm weighes about 1/5 of the female worm (unpublished data). We could detect AChE activity in 2ml of culture medium with 25 adult female worms. However, we could not detect the secretion of AChE by adult male worms. The inability of adult male worms to secrete AChE in contrast to females may leave the posibility that AChE secreted by adult female worms came from microfilariae, because a number of microfilariae were released in culture medium. In our experiments, about 300 microfilariae were released from a adult female worm in Eagle's MEM for 24 hours. However, the AChE activity was not determined in the culture fluid with 100,000 microfilariae. This result strongly supports the idea that AChE is secreted from adult female worms. Rathaur *et al.* (1987) demonstrated the secretory AChE in the culture medium where *B. malayi* adult female worms, male worms or microfilariae were cultured. In their study, the activity of AChE was measured by a microplate adaptation of the procedure of Ellman *et al.* (1961). Therefore, their results raise the question whether the enzyme might be secreted by *B. pahangi* male worms at a level which could not detected by Ellman's technique (1961) used in the present study.

AChE secretion by adult female worms was influenced by the medium used for culture of worms, number of worms in a given volume of medium, and the period of incubation. Of the medium used in our experiments, only Eagle's MEM stimulated the secretion of AChE. In HBSS and PBS(−), the worms became less active rapidly. In Eagle's MEM, the worms could be maintained alive for 20 days. However, the movement of the worms became somewhat slower during the incubation. When 150 female worms were cultured in 2ml of medium, they became less active during the 3−4 days in culture. These results suggest that AChE secretion is closely related to the activity of the worms cultured. Filarial worms are likely to be more active in vitro than they are when they are first removed from the animal host (Taylor, 1960), and this increased activity may account for the sharp rise in AChE secretion during the first 24 hours in culture.

AChE secreted by *B. pahangi* adult female worms was identified as a true AChE, because it hydrolysed acetylthiocholine 4 times faster than butyrylthiocholine and propionylthiocholine, was inhibited by eserine, and disc electrophoresis disclosed a single band corresponding to the relative mobility of the authentic AChE from electric eel. Regarding the glands or organs which secrete the AChE, our histochemical studies on *B. pahangi* larvae have revealed that AChE activity was localized in several non−nervous tissues; amphids, phasmids and glandular region of oesophagus (Nakajima *et al.*, 1985). It is, therefore, highly probalbe that AChE is secreted from amphidial glands as in the case of *Necator americanus* (McLaren, 1976). However, it is also possible that AChE is secreted from female worms when microfilariae are released.

It is well known that some gut−dwelling nematodes secrete unusually high level of

AChE *in vitro*. For these nematodes, AChE is located in the oesophageal and excretory glands (Rothwell *et al.*, 1973). Although the biological role of AChE in the host-parasite relationship is as yet undetermined, following possibilities have been proposed. The enzyme functions as a biological holdfast, allowing the worms to maintain their position amongst the intestinal microvilli (Ogilvie and Jones, 1971). The enzyme alters the permeability of the host membrane and thus facilitates the leakage of nutrients (Lee, 1970). The enzyme interferes with the host immune response by degrading acetylcholine, which itself enhances the immunologically triggered release of mediaters by mast cells and cytotoxicity by neutrophils and lymphocytes (Phoads, 1984). Recently, AChE was proved to be secreted from non gut−dwelling nematodes; *Stephanurus dentatus*, a nematode which infects the kidneys of swine (Rhoads, 1981), *B. malayi* (Rathaur *et al.*, 1987) and *B. pahangi* (present communication). These novel findings are probably indicative of an important role of secretory AChE in host−parasitic nematode relationship.

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