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Original article

**Characterization of acetylcholinesterase secreted
by the trichostrongyle nematode parasites
of ruminants**

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Summary — Acetylcholinesterase (AChE) secreted by seven different ruminant trichostrongyles was studied in vitro. AChE activity was particularly high (84 and $160 \times 10^{-3} \text{ M}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$) in the excretion products of *Nematodirus spathiger* and *N battus*, moderate (3 and $5 \times 10^{-3} \text{ M}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$) for *Trichostrongylus colubriformis* and *T vitrinus* and low ($0.9 \times 10^{-3} \text{ M}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$) for *Teladorsagia circumcincta*. No activity was observed with *Haemonchus contortus* and *Cooperia curticei*. At 4°C , 80% of AChE activity was maintained over 72 h except for *T circumcincta* where a loss of 50% was observed after 24 h. At 37°C , *N spathiger* and *T colubriformis* maintained an activity over 72 h, but for the other species, a loss of 50% was observed after 24 h. The molecular weights of the AChE from the different species, estimated by gel filtration (Sephadex S300HR), ranged between 64 and 150 kDa. The coefficients of sedimentation estimated by sucrose density gradient ranged between 4.8 S and 7.8 S and corresponded to a monomeric hydrophilic form (G1). For *T vitrinus*, an amphiphilic form was suspected.

acetylcholinesterase / excretion secretion / trichostrongyle nematode / ruminant

Résumé — Caractérisation de l'acétylcholinestérase sécrétée par les trichostrongyles, nématodes parasites de ruminants. L'acétylcholinestérase (AChE) sécrétée in vitro a été étudiée chez sept espèces de nématodes parasites des ruminants. L'activité de l'AChE est particulièrement élevée (84 et $160 \times 10^{-3} \text{ M}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$) dans les produits d'excrétion/sécrétion de *Nematodirus spathiger* et *N battus*, modérée (3 et $5 \times 10^{-3} \text{ M}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$) pour *Trichostrongylus colubriformis* et *T vitrinus* et faible ($0.9 \times 10^{-3} \text{ M}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$) pour *Teladorsagia circumcincta*. Aucune activité n'a été observée pour *Haemonchus contortus* et *Cooperia curticei*. A 4°C , 80 % de l'activité de l'AChE est maintenue pendant 72 heures sauf pour *T circumcincta* où, dès 24 heures, il y a une perte de 50 %. A 37°C , *N spathiger* et *T colubriformis* maintiennent une activité pendant 72 heures alors que les autres espèces perdent 50 % d'activité dès 24 heures. Le

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poids moléculaire de l'AChE, estimé par chromatographie sur Sephadex S300HR, varie de 64 à 150 kDa suivant les espèces. Le coefficient de sédimentation, déterminé par centrifugation sur gradient de saccharose varie de 4.8 S à 7.8 S, ce qui correspond à un monomère de type hydrophile (G1). Pour *T. vitrinus*, une forme amphiphile peut être suspectée.

acétylcholinestérase / excrétion sécrétion / nématode trichostrongylidé / ruminant

INTRODUCTION

Trichostrongyle parasites in the gastrointestinal tract of domestic ruminants are known to induce important pathophysiological changes in their host (Castro, 1981; Parkins and Holmes, 1989). Whether these changes are caused directly by the worms or indirectly through the host reaction or modifications of the host regulatory system remains unclear. Concerning the direct effect of the worms, evidence exists for a mechanical effect. However, a role of the excretion and secretion (ES) products released by the nematodes in the local environment is also suspected and some in vitro results have reinforced this hypothesis (Mc Kellar et al, 1990; Hoste et al, 1995).

Amongst the ES products, acetylcholinesterase (AChE) has received particular attention as a role for this enzyme in host-parasite relationships is widely suspected (Pritchard, 1993) and this enzyme has been shown to be secreted in vitro by many nematode species (Ogilvie et al, 1973). Nevertheless, some ruminant parasite species have not been examined and the ES products of the worms have only been collected in physiological saline and for short time intervals. It is therefore difficult to refer to these results for any studies on worm physiology which suppose a long term maintenance of the nematodes in a more appropriate medium. On the other hand, it has been suggested that differences in the molecular form of secreted AChE by nematodes may influence the effects of the ES products on the digestive tract. However, amongst the ruminant nematode parasites, the purification and biochemical character-

isation of the AChE secreted has only been achieved for *T. colubriformis* (Griffith and Pritchard, 1994) and it appears important to assess whether differences in the enzyme activities among trichostrongyle species are also related to molecular differences in the secreted AChE.

In this paper we compared the AChE secreted over 48 h in vitro in a defined medium by seven species of nematode parasites from the gastrointestinal tract of sheep: *Nematodirus battus*, *Nematodirus spathiger*, *Teladorsagia circumcincta*, *Haemonchus contortus*, *Cooperia curticei*, *Trichostrongylus vitrinus* and *T. colubriformis*.

MATERIALS AND METHODS

Collection of parasites and ES products

For each species, worm free sheep were infected with 10 000 infective larvae. Twenty-one days after infection, the adult parasites were removed in physiological saline by the Baermann technique from the appropriate part of the gastrointestinal tract: the sheep intestine for *N. battus*, *N. spathiger*, *C. curticei* and *T. vitrinus*, the sheep abomasum for *T. circumcincta* and *H. contortus* or the rabbit intestine for *T. colubriformis*. The worms were washed three times in Dolbecco modified Eagle medium (DMEM) containing streptomycin (1 mg/mL) and penicillin (1 000 U/mL) in order to avoid any contamination by host material. The number of worms was adjusted to a concentration of 200 worms/mL (Bone and Bottjer, 1986), except for the *Nematodirus* species and *H. contortus*, (100 worms/mL). All worm samples were then maintained for 48 h in 25 mL tissue culture flasks in DMEM at 37 °C in a humidified atmosphere of air and CO₂ (95%/5%). The medium was changed after 24 h. During this period, the cul-

tures were routinely observed and those with less than 90% of the worms motile were discarded in order to prevent contamination with non-secretory AChE. After worm sedimentation, the culture supernates were collected and filtered through low-protein binding 0.22 µm filters (Millex GV, Millipore SA, Molsheim, France). The supernates were then immediately frozen in liquid nitrogen and stored at -70 °C.

Protein estimation and determination of AChE activity

The protein concentration of the ES products was estimated by the microassay procedure of the Bio-Rad Protein Assay (Bio-Rad laboratories GmbH, München, Germany). The assay was performed using a micro-method with 50 µL of reagent (Coomassie Brilliant Blue G-250). For each batch of worms, 0.2 mL of culture medium was tested as well as dilutions (3-25 µg/mL) of bovine serum albumin (Sigma SA) as the standard.

The AChE activity was assayed by the colorimetric technique of Ellman et al (1961) using a micromethod with 20 µL samples. The test was performed using acetylthiocholine iodide (ATI) (Sigma SA) as substrate. Butyrylthiocholine iodide (BTI) (Sigma SA) was substituted as a substrate to establish the substrate specificity of the enzyme.

To test the stability of AChE at different temperatures, aliquots of the ES products containing AChE were stored at 4 and 37 °C. The AChE activity was tested after 24, 48 and 72 h of conservation.

Sedimentation analyses in a sucrose gradient

Apparent sedimentation coefficients (S) were determined on 5-20% sucrose gradients in high salt (HS) or high salt with Brij 96 detergent (HSB) buffers. The HS buffer consisted of 20 mM Tris-HCl pH 7.5, 1 M NaCl, 5 mM MgCl₂, 0.1 mg/mL bacitracin, 0.008 TUI/mL aprotinin and 1 mM EDTA. HSB consisted of HS with 1% Brij 96 (10 oleylether Sigma SA). Marker proteins were included: *E coli* B-galactosidase, 16S (Boehringer SA) and alkaline phosphatase, 6.1S (Sigma SA). Sedimentation was

performed at 4 °C with a SW41 rotor at 200 000 g for 18 h in a Beckman XL70 ultracentrifuge. Fractions of 0.3 mL were then collected from the bottom of the tube and tested for AChE activity.

Chromatographic fractionation

Gel filtration of the AChE from the ES cultures was performed using samples of 1.5 mL on a Sephadex S-300 HR column (Sigma SA), with a bed height of 65 cm and a diameter of 1.5 cm. The column was equilibrated in PBS, pH 7.2 at a flow rate of 0.3 mL/min at 4 °C; 1.5 mL fractions were collected. Blue dextran (2 000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine albumin (66 kDa) and carbonic anhydrase (29 kDa) were used as the molecular weight standards for calibration (Sigma SA).

RESULTS

Protein and acetylcholinesterase (AChE) secretion

Table I shows the level of AChE and proteins in the ES products of the different species. Protein secretion remained constant for 48 h in vitro while AChE secretion during the first 24 h was twice the value observed during the second day of worm maintenance.

The amount of AChE secreted was different between species. Basically, three groups of worms could be distinguished by their amount of AChE secretion. A particularly high AChE activity was observed in the secretions of the two *Nematodirus* species. Moderate to low levels of AChE were observed in the ES products from *Trichostrongylus* species and *T. circumcincta*. Finally, no activity was detected in either the *H. contortus* or the *C. curticei* ES products.

The secretions of each species hydrolysed the substrate BTI at a rate of no more than 20% of ATI hydrolysis.

Table I. Levels of acetylcholinesterase and protein secreted by the worms in vitro.

	Protein ^a		AChE ^b		BChE/AChE %
	24 h	48 h	24 h	48 h	
<i>N battus</i>	0.20 ± 0.05 (5)	0.18 ± 0.04 (5)	160 ± 76 (5)	89 ± 24 (5)	12
<i>N spathiger</i>	0.26 ± 0.02 (2)	0.19 ± 0.02 (3)	84 ± 6 (2)	34 ± 5 (3)	21
<i>T colubriformis</i>	0.05 ± 0.01 (4)	0.03 ± 0.01 (4)	3.4 ± 2.3 (4)	1.0 ± 0.5 (4)	14
<i>T vitrinus</i>	0.04 ± 0.02 (3)	0.02 ± 0.01 (3)	5.0 ± 1.8 (3)	2.0 ± 0.8 (3)	20
<i>C curticei</i>	0.04 ± 0.01 (3)	0.04 ± 0.01 (2)	not detected	not detected	–
<i>H controtus</i>	0.21 ± 0.03 (2)	0.24 ± 0.01 (2)	not detected	not detected	–
<i>T circumcincta</i>	0.14 ± 0.03 (3)	0.10 ± 0.01 (2)	0.9 ± 0.3 (3)	0.3 ± 0.1 (2)	2

^a µg/worm^b Moles of acetylthiocholine hydrolysed/g of protein/min ($\times 10^{-3}$) at 37 °C and pH 7 mean ± SD; () number of samples tested.**Stability of AChE activity in ES products**

At 4 °C, (table II) after 48 h of storage, about 80% of the AChE activity was maintained in the ES products of *N battus*, *N spathiger*, *T colubriformis* and *T vitrinus*. For the ES products from *T circumcincta*, a 50% loss in AChE activity was observed within the first 24 h.

At 37 °C, only *N spathiger* and *T colubriformis* maintained a high AChE activity after 48 h of storage. With the other species,

a loss of about 50% was observed after the first 24 h.

Molecular forms of AChE

On sucrose density gradients (fig 1), the AChE of the ES products resolved as a single peak at 5.1 S for *N battus*, 4.9 S for *N spathiger*, 4.8 S for *T circumcincta* and 6.1 S for *T colubriformis*. For these species, no

Table II. Temperature stability of AChE activity ($\text{mol}\cdot\text{min}^{-1}\cdot 10^{-5}$)

	T 0		24 h		48 h		72 h	
	4 °C	37 °C	4 °C	37 °C	4 °C	37 °C	4 °C	37 °C
<i>N battus</i>	68	68	53	35	50	28	27	16
<i>N spathiger</i>	71	71	58	55	58	56	59	54
<i>T colubriformis</i>	3.7	3.7	3.0	2.8	3.0	2.5	2.5	2.0
<i>T vitrinus</i>	2.8	2.8	2.4	1.6	2.3	1.5	2.2	1.3
<i>T circumcincta</i>	4.2	4.2	2.0	1.5	1.8	1.5	1.5	1.5

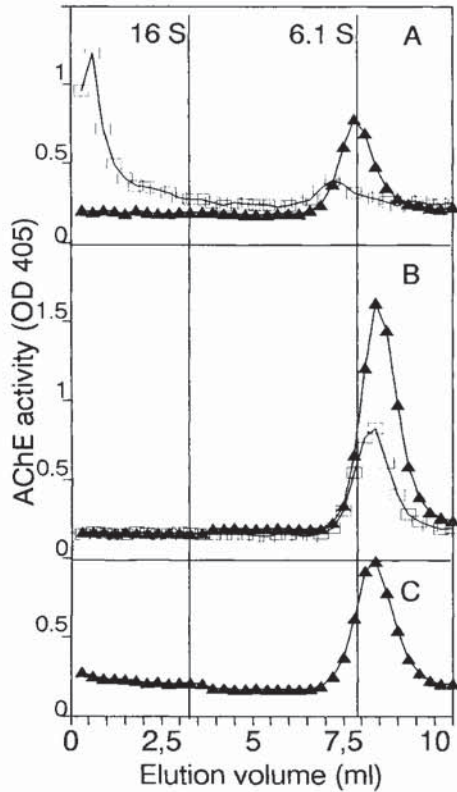


Fig 1. Sucrose density gradient of ES products run using HS buffer at 200 000 *g* for 18 h at 4 °C. A: *Trichostrongylus colubriformis* (black triangle), *T vitrinus* (open square); B: *Nematodirus spathiger* (black triangle), *N battus* (open square); C: *Teladorsagia circumcincta*. Vertical lines: position of internal standards of 16 S (B-galactosidase) and 6.1 S (alkaline phosphatase)

shift in sedimentation was observed in the HSB gradients (not shown), confirming the presence of hydrophilic globular (G) forms in these species. A different pattern was observed for the *T vitrinus* ES products with a small peak at 7.8 S and most of the AChE activity concentrated in the first fractions, which corresponded to a sedimentation coefficient above 16 S. The 7.8 S sedimentation peak shifted to 6.8 S in the HSB gradient

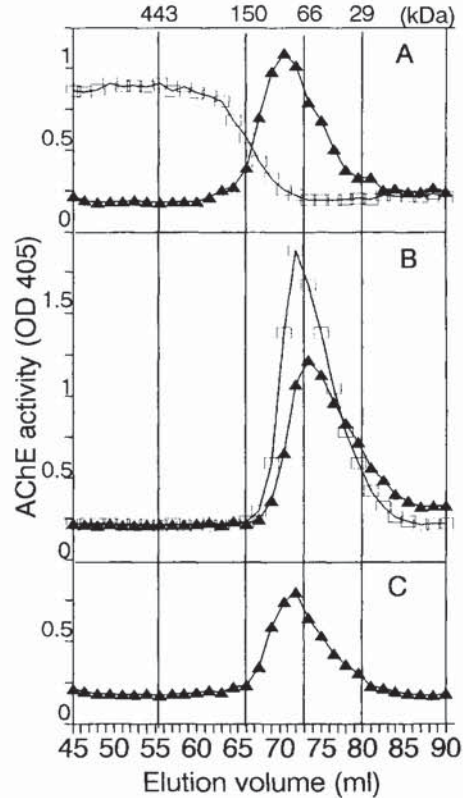


Fig 2. Gel filtration of ES products using Sephadex S-300 HR. A: *Trichostrongylus colubriformis* (black triangle), *T vitrinus* (open square); B: *Nematodirus spathiger* (black triangle), *N battus* (open square); C: *Teladorsagia circumcincta*. Vertical lines: position of the standards of 433 kDa (apoferritin), 150 kDa (alcohol dehydrogenase), 66 kDa (bovine albumin), and 29 kDa (carbonic anhydrase).

(not shown), suggesting the presence, in this species, of an amphiphilic form.

Using gel filtration on Sephadex S300 (fig 2), the AChE activity was eluted in single peaks corresponding to molecular weights of 69 kDa for *N battus*, 64 kDa for *N spathiger*, 75 kDa for *T circumcincta* and 84 kDa for *T colubriformis*. For *T vitrinus*, a constant level of AChE activity was eluted from dead volume to a molecular weight of 150 kDa.

DISCUSSION

Most of previous research comparing the secretion of AChE in trichostrongyle species (Ogilvie et al, 1973; Rothwell et al, 1973; Knox and Jones, 1990) was performed using physiological saline for periods not exceeding 8 h. Others have used Jenkins medium (Jenkins et al, 1980) which has proven to be more suitable for long-term in vitro studies but these studies concerned only some peculiar species, ie, *T colubriformis* (Griffith and Pritchard, 1994) or *Heligmosomoides polygyrus* (Mallet, 1989). The present results are the first to compare the persistence of AChE secretion in several trichostrongyle species over a 48 h period in DMEM, a well-defined medium.

Most AChE activity was observed in the ES products from the *Nematodirus* species with a rate of excretion 30 times higher than *T colubriformis* which was previously considered to be one of the highest AChE producers (Rothwell et al, 1973). This result agreed with a previous study where very high amounts of AChE activity were detected in *N battus* homogenates (Lee and Martin, 1976), but contrasted with another experiment where no activity was detected in the ES products of the same species incubated in physiological saline (Knox and Jones, 1990). The use of a medium more suitable for worm survival in the present experiment may explain such a difference.

Four of the intestinal species examined in the present study (*N battus*, *N spathiger*, *T colubriformis* and *T vitrinus*) secreted high levels of AChE but no activity was detected in ES products of the fifth species (*C curticei*). For the two species from the abomasum, low levels of AChE activity were observed in ES products from *T circumcincta* but none from *H contortus*. Thus, no clear correlation was observed between the level of AChE secretion and the part of the gastrointestinal tract inhabited by the worms.

As previously observed with AChE secreted by some nematode species (Grif-

fith and Pritchard, 1994), the enzyme in ES products hydrolyses the substrate acetylthiocholine iodide at a higher rate than butyrylthiocholine iodide suggesting that the tested activity corresponds to the true AChE activity (EC 3.1.1.7). As in other invertebrates, it is likely that this enzyme is responsible for the hydrolysis of butyrylthiocholine iodide.

If a molecule is to be active in vivo, it has to be stable at least at physiological temperatures. A significant decrease was observed in the AChE activity of ES products stored for 24 h at 37 °C. This decrease, which varies between species, may be explained by differences in the proteinase content of the ES products. Such proteinase activities have been previously detected in ES products of various nematode species (Knox and Jones, 1990). Nevertheless, the stability of AChE may be better in vivo than in vitro where regular secretion may maintain a constant level of enzyme locally.

The molecular analysis of secretory AChE has been previously described for *Nippostrongylus brasiliensis* (Blackburn and Selkirk, 1992) and *Necator americanus* (Pritchard et al, 1991). In purified AChE from *T colubriformis* ES products (Griffith and Pritchard, 1994), two molecular forms have been observed corresponding to a globular monomeric (G1) and a dimeric (G2) molecular form. The molecular weight of these two proteins was estimated to be 80 and 189 kDa respectively. In the present study, AChE from the same species resolved by gel filtration in a single peak with a molecular weight of 84 kDa. This corresponded to the G1 form and no activity was observed corresponding to the G2 form previously observed. This is not really surprising as the G2 form was observed on highly purified and concentrated enzyme preparations with a lower activity than the G1 form and was not detected by sucrose density gradient or non denaturing electrophoresis. If the G2 form exists in native ES products, it

is certainly at a low concentration and cannot be detected by the methods applied in the present study. A similar pattern was observed in the present experiment with *T. circumcincta* and *Nematodirus* spp. but with slight differences in molecular weight. Such differences between the AChE from different species have been previously observed. They could be explained by differences in the glycosylation of the molecule (Massoulié et al, 1993).

The aggregation of the AChE secreted by *T. vitrinus* observed on sedimentation and gel filtration profiles as well as the shift from 7.8 S to 6.8 S in the presence of the detergent Brij 96 suggested that *T. vitrinus* secretes an amphiphilic form of AChE which is different from the hydrophilic forms generally observed in nematode ES products. Secretion of amphiphilic AChE has already been reported in some instances (Massoulié et al, 1993).

In conclusion, we observed that within the tested species, different amounts of AChE were secreted in vitro. The role of this enzyme in host parasite relationships has now to be ascertained, especially for the high secreting species.

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