Multiple molecular forms of cholinesterase in the plant-parasitic nematodes *Meloidogyne incognita* and *Radopholus similis* [(1)]

Dieter NORDMEYER* and Don W. DICKSON

SUMMARY

Five different molecular forms of cholinesterase (ChE) from *Meloidogyne incognita* and four different forms from *Radopholus similis* were separated by velocity sedimentation. Three of the forms appeared common to both nematode species. Two forms (I and II) found in *M. incognita* second-stage juveniles were much less active in females. The addition of sodium deoxycholate increased solubility of forms I and II, whereas form III was inhibited slightly. The nonionic detergent Triton X-100 strongly inhibited all ChE activity. Comparative sensitivities of total ChE from *M. incognita* and *M. javanica* to five nonfumigant nematicides were very similar, whereas acetylcholinesterase from electric eel behaved differently. Eserine was a potent inhibitor of ChE from *M. javanica.*

RÉSUMÉ

Formes moléculaires multiples de l'acétylcholinestérase chez les nématodes parasites des plantes Meloidogyne incognita *et* Radopholus similis

The nematodes used were *Meloidogyne incognita* (Kofoid & White) Chitwood, *M. javanica* (Treub) Chitwood, *Radopholus similis* (Cobb) Thorne, and *R. citrophilus* (Huettel, Dickson & Kaplan). *Meloidogyne incognita* and *M. javanica* were cultured in the greenhouse on tomato (*Lycopersicon esculentum* Mill. cv. Rutgers). Nematode eggs were extracted from tomato roots using 0.5 % sodium hypochlorite (Vrain, 1977) and cleaned from debris by a centrifugation-sugar-flotation technique (Jenkins, 1964). The eggs were hatched on a 17 µm nylon screen. Freshly hatched second-stage juveniles (not older than 48 hours) were used for the experiments. Nematode females were extracted from tomato roots using Pectinol 59L (Hussey, 1971). *Radopholus similis* and *R. citrophilus* were maintained at 26° C on carrot disc cultures (Huettel, 1982). Nematodes (mixed-life stages) of these two species were washed from the sides of culture tubes and aerated 24 hours in water before being isolated for maceration. Nematodes were macerated in 1.0 ml ice-cold 0.05 M borate buffer, pH 8.8 in a glass tissue homogenizer with glass beads (0.1 mm diam.).

Analytical velocity sedimentations for separations of multiple cholinesterase (ChE) forms were carried out as described by Johnson and Russel (1983). Sample aliquots of 100 µl were layered on top of a 4.5 ml, 5-20 % sucrose gradient containing borate buffer, with an underlying 0.5 ml cushion of Renografin. The gradient was then centrifuged at 4° C for 5 hours at 48 000 rpm in a SW 50.1 rotor (Beckman L5-50 ultracentrifuge). Fractions of five drops each were collected by puncturing the bottom of the centrifuge tube. Gradients generally yielded 22-24 fractions. Parallel gradients of the three sedimentation marker proteins, B-galactosidase, catalase and alkaline phosphatase, were also run. All analyses were repeated five to seven times.

ChE assays were routinely conducted at room temperature (± 25° C) by the radiometric single-vial liquid-extraction method (Johnson & Russell, 1975) which was modified slightly. Reaction volumes of 100 µl in a 1-dram scintillation vial contained 1×10^{-6} M (^{3}H-acetyl)-choline, 25 mM HEPES buffer, pH 7.0, and 10-25 µl of the nematode fraction to be assayed. Assays were initiated by addition of the nematode fraction and stopped by addition of 100 µl of a « stopping solution » containing 1.0 M monochloroacetate buffer, pH 3.0 and 2.0 M NaCl. Four milliliters of scintillation fluid (0.5 % PPO, 0.03 % POPOP in toluene — 10 % vol/vol 1-butanol) were then added and vials were capped, shaken vigorously, and counted in a liquid scintillation counter. Duplicate assays were run on each sample. Each set of assays included several blanks and complete conversions (with electric eel AChE) as controls. All counts were corrected for background and quenching and reported as counts per minute per millimole. Catalase, B-galactosidase, and alkaline phosphatase were assayed as described by Johnson and Russell (1983), Miller (1972) and Hausamen *et al.* (1967), respectively. Fractional positions of assay peaks were calculated by the formula : $(n - x)/n$, in which n is the total number of fractions from the bottom of the tube (Johnson & Russell, 1983).

Molecular forms of ChE were analyzed from 80 000 second-stage juveniles (J2), or 10 000 females of *M. incognita*, or 60 000 individuals of mixed life stages of *R. similis*. Each preparation was homogenized in 1 ml ice-cold borate buffer and incubated for 24 hours at 5° C. The supernatants were loaded onto a sedimentation gradient as described. Individual fractions (25 µl) were assayed for ChE activity for 10 minutes at room temperature (25° C).

Solubility properties of ChE were determined from *M. incognita* (J2). Fifty thousand J2 were homogenized in 1 ml borate buffer and incubated for 24 hours at 5° C. The homogenate was centrifuged in a Beckman Airfuge at 100 000 *g* for 20 minutes at 5° C, the supernatant was removed and the pellet was resuspended three times in buffer and centrifuged. The pooled fractions were loaded onto the sedimentation gradient and ChE activity determined as described. In a second solubilization procedure, the homogenate in standard borate buffer was mixed with DOC (final concentration 0.2 %) for 5 minutes at room temperature (25° C). In a third tration 1 %). The homogenate was then loaded on a sedimentation gradient and centrifuged as described. From the fractions collected, 50 µl were assayed for 5 minutes at room temperature (25 °C). In a third solubilization procedure, the homogenate in borate buffer was mixed with Triton X-100 (final concentration 0.1 %) for 24 hours. Then it was loaded on a sedimentation gradient and treated for ChE determination as described. The detergent was removed from the preparations by adding 0.3 g SM-2 Bio-Beads per ml of preparation.

Inhibition of ChE from *M. incognita* and *M. javanica* J2 by aldicarb, carbofuran, ethoprop, fenamiphos, and oxamyl were compared. The nematodes (50 000 J2) of each species were homogenized in 1 ml borate buffer, incubated for 24 hours at 5° C, and centrifuged at 100 000 *g* for 20 minutes at 5° C. The clear supernatant was used for ChE inhibition studies with the five different nematicides and eserine. Stock solutions (1000 µg/ml) of each nematicide were prepared in acetone and stored at 5° C no longer than 14 days. AChE from electric eel was included for comparison. After addition of the inhibitor, 10 µl of the enzyme were assayed for 10 minutes at room temperature as described. The I_{50} (nematicide concentration that inhibited 50 % of the nematode ChE) was determined by assaying different concentrations of each nematicide. The degree of inhibition was determined in percent and the I_{50} values was calculated for each treatment.

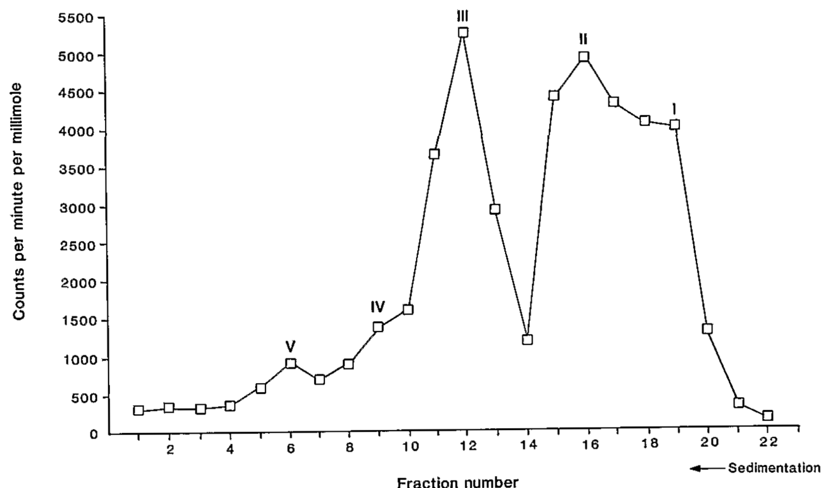

Fig. 1. Five multiple molecular forms of cholinesterase from *Meloidogyne incognita* second-stage juveniles that were separated by velocity sedimentation. The ChE forms were designated with Roman numerals beginning from the top of the sedimentation gradient. Each figure is the mean of at least five analyses.

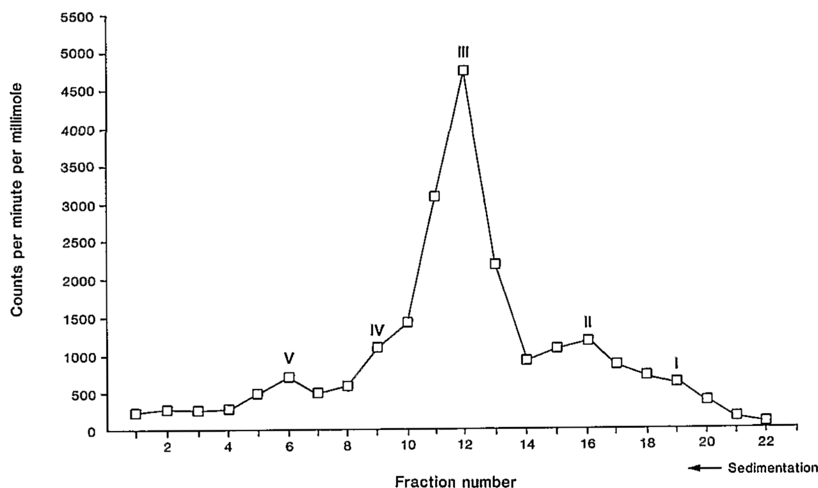

Fig. 2. Five multiple molecular forms of cholinesterase from *Meloidogyne incognita* females that were separated by velocity sedimentation. The ChE forms were designated with Roman numerals beginning from the top of the sedimentation gradient. Each figure is the mean of at least five analyses.

Results and discussion

Five different molecular forms of ChE were demonstrated for *M. incognita* and *M. javanica* (Figs 1, 2) and four for *R. similis* and *R. citrophilus* (Fig. 3). The ChE forms separated from *M. incognita* and *M. javanica* showed no visible differences as was also the case for forms separated from *R. similis* and *R. citrophilus* (data not shown). Five ChE bands from *Meloidogyne* spp. were also reported from comparisons made by concentration gradient PAGE and ultracentrifugation analysis (Premachandran *et al.,* 1982).

Our ChE forms were designated with Roman numerals beginning from the top of the sedimentation gradient. Fractional positions of ChE forms were considered to be accurate indicators for comparisons of

different molecular forms of ChE among nematode species or developmental life stages of the same species (Table 1), because all experiments were conducted under identical conditions and were repeated five to seven times with little variation of results (always compared to the three marker proteins). Based on the fractional position of the five molecular forms of ChE, both developmental stages of *M. incognita* showed identical ChE forms (Figs 1, 2). But forms I and II from females were much less active than those same forms in the J2. Attempts to increase solubility of female ChE by increasing incubation times or by the use of detergents did not yield significantly more ChE (data not shown). Forms IV and V showed little activity in both developmental stages of the nematode. Whether these molecular forms of ChE are in part identical to those reported from *C. elegans* (Johnson & Russell, 1983) is not known. Such comparisons were not a part of this study.

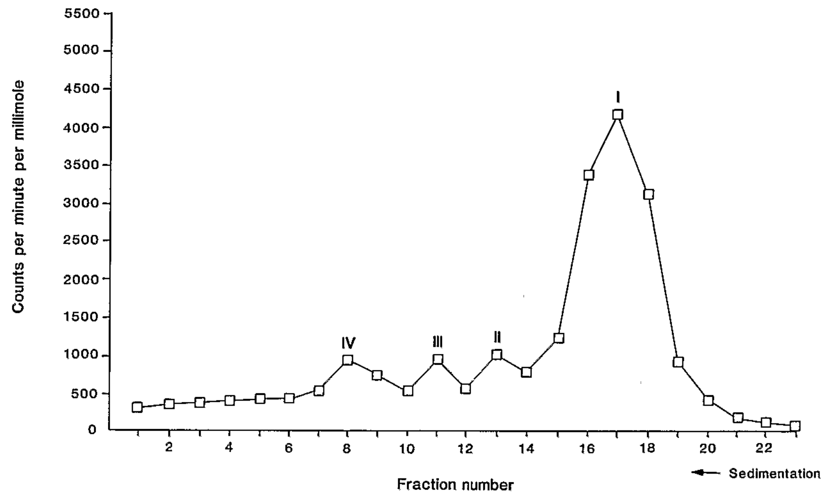

Fig. 3. Four multiple forms of cholinesterase from *Radopholus similis* (mixed developmental stages) that were separated by velocity sedimentation. The ChE forms were designated with Roman numerals beginning from the top of the sedimentation gradient. Each figure is the mean of at least five analyses.

Table 1

Fractional positions of five molecular forms of cholinesterase (ChE) from *Meloidogyne incognita* and four molecular forms of ChE from *Radopholus similis* separated by velocity sedimentation.

	M. incognita ChE forms					*R. similis* ChE forms				Marker proteins		
	I	II	III	IV	V	I	II	III	IV	Alkaline phosphatase	Catalase	B-Galactosidase
Sedimentation Fractional Position*	0.272 ± 0.004	0.322 ± 0.004	0.519 ± 0.006	0.630 ± 0.005	0.742 ± 0.009	0.317 ± 0.003	0.433 ± 0.005	0.521 ± 0.006	0.626 ± 0.008	0.29	0.57	0.69

* Data are means of five analyses and the standard deviation for each molecular form of ChE is shown. Assay peak positions were converted to fractional positions by the formula : $(n - x)/n$, in which n is the total number of fractions collected (to the nearest 0.25 fraction), and x is the fraction number of the assay peak (fractions numbered beginning from the tube bottom; see Johnson & Russell, 1983).

Radopholus similis had four ChE forms (Fig. 3) of which three appeared identical or at least very similar to those in *M. incognita.* Two forms (I & V) in *M. incognita* were absent in *R. similis* and one form (II) in *R. similis* was absent in *M. incognita.* The dominant forms in each nematode seemed identical. Form I from *R. similis* had a fractional position of 0.317 and form II from *M. incognita* was 0.322 (Table 1). Because of the great reduction in activity of forms I and II in *M. incognita* females, it is possible that these forms are essential and involved in specific functional roles, e. g. involvement in hydrolysis of acetylcholine at nerve synapses that control body

movement. Once the nematode becomes sedentary, the production of forms I and II may be reduced or turned off. The form with a similar functional role in adult migratory nematodes would likely remain active such as we recorded in *R. similis.* Comparative studies are needed however, with other sedentary and migratory nematode species to further elucidate this possibility. AChE studies on *C. elegans* indicate that different classes of AChE may have different functions within the nematode nervous system (Johnson *et al.,* 1988). It is possible that this is true also for plant-parasitic nematodes.

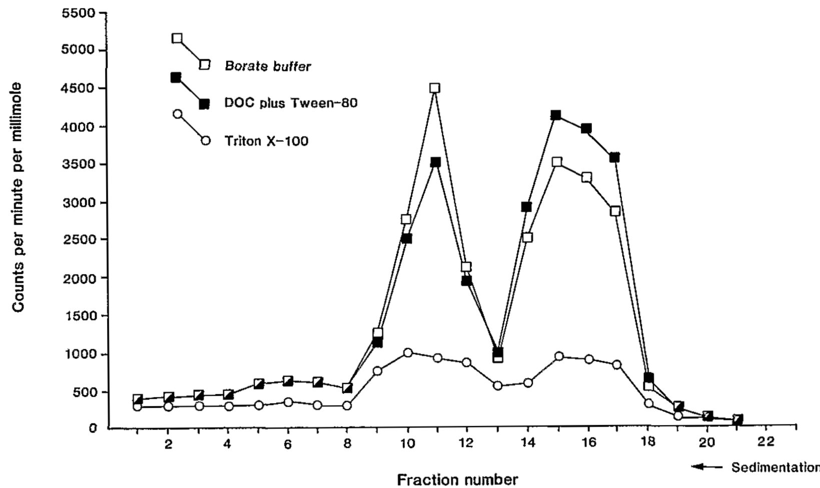

Fig. 4. Effect of two detergents, sodium deoxycholate (DOC) (0.2 %) plus Tween 80 (1 %), and Triton X-100 (0.1 %) on solubility of cholinesterase from *Meloidogyne incognita* second-stage juveniles compared with standard borate buffer treatment. Cholinesterase activity was separated by velocity sedimentation.

The addition of DOC to our preparations increased the solubility of forms I and II in *M. incognita,* whereas form III was inhibited slightly (Fig. 4). The use of DOC also increased the solubility of form I in *R. similis.* The use of the nonionic detergent Triton X-100 strongly inhibited all ChE activity in *M. incognita.* The differential sensitivity to DOC between forms I and II from *M. incognita* compared with form III could indicate that these forms represent different classes of ChE. Johnson and Russell (1983) reported two classes (A and B) of AChE from *C. elegans* based on their solubility properties and subsequently a third class (C) was reported (Kolson & Russell, 1985*a, b;* Johnson *et al.,* 1988).

Comparisons of sensitivity of total ChE from *M. incognita* and *M. javanica* with AChE from electric eel showed that both *Meloidogyne* species exhibited similar ($P < 0.05$) sensitivity towards various inhibitors (Tab. 2). AChE from electric eel, however behaved differently in that it was somewhat more sensitive ($P < 0.05$) to fenamiphos and aldicarb, but less sensitive to ethoprop and to eserine. Eserine was an extremely potent inhibitor of *M. javanica* ChE. There was great sensitivity of ChE from both *M. incognita* and *M. javanica* to the carbamates, aldicarb, carbofuran or oxamyl and much less to the organophosphates, ethoprop or fenamiphos. Also, the inhibition studies (Table 2) indicate identical or close resemblance of ChE from these two *Meloidogyne* species.

Table 2

Sensitivity of cholinesterase (ChE) obtained from *Meloidogyne incognita, M. javanica* and acetylcholinesterase from electric eel to several ChE inhibitors.

	I_{50} ChE (1×10^{-6} M)*		
Inhibitor	*M. incognita*	*M. javanica*	Electric eel
Fenamiphos	875 *a*	980 *a*	324 *b*
Ethoprop	555 *a*	483 *a*	980 *b*
Aldicarb	3.1 *a*	3.1 *a*	0.9 *b*
Oxamyl	0.17 *a*	0.20 *a*	0.24 *a*
Carbofuran	0.02 *a*	0.02 *a*	0.02 *a*
Eserine	**	0.002 *a*	0.02 *b*

Data are means of three experiments and each analyses was run as a duplicate set. Means across columns with the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).

* Concentrations that inhibited 50 % of the ChE or AChE.

** Not determined.

ACKNOWLEDGEMENTS

We thank Dr. C. D. Johnson for his helpful advice and support during this study and also Dr. R. N. Huettel for supplying the cultures of *Radopholus similis* and *R. citrophilus.* We thank DuPont, FMC, Mobay, Mobil, and Union Carbide for providing technical formulations of nematicides and partial support of this investigation.

REFERENCES

CRIBBS, L. L. (1983). *Interrelationships of the multiple molecular forms of acetylcholinesterase in the nematode,* Caenorhabditis elegans. Ph. D. Diss., Univ. Pittsburgh, PA, USA, 111 p.

CUANY, A., BERNIER, F., BRIDE, J. M., WALLACH, J. & BRIDE, J. B. (1981). Caractérisation des ChE chez deux nématodes : action inhibitrice de l'aldicarbe et de ses dérivés d'oxydation. *Trav. Soc. Pharmacie Montpellier,* 41 : 147-151.

CULOTTI, J. G., VON EHRENSTEIN, G., CULOTTI, M. R., & RUSSELL, R. L. (1981). A second class of acetylcholinesterase-deficient mutants of the nematode *Caenorhabditis elegans. Genetics,* 97 : 281-305.

HAUSAMEN, T. U., HELGER, R., RICK, W., & GROSS, W. (1967). Optimal conditions for the determination of serum alkaline phosphatase by a new kinetic method. *Clin. Chem. Acta,* 15 : 241-245.

HUETTEL, R. N. (1982). *Genetic bases for identification and separation of the two Florida races of* Radopholus similis

JOHNSON, C. D., DUCKETT, J. G., CULOTTI, J. G., HERMAN, R. K., MENEELY, P. M. & RUSSELL, R. L. (1981). An acetylcholinesterase-deficient mutant of the nematode *Caenorhabditis elegans. Genetics,* 97 : 261-279.

JOHNSON, C. D., RAND, J. B., HERMAN, R. K., STERN, D. & RUSSELL, R. L. (1988). The acetylcholinesterase genes of *C. elegans* : Identification of a third gene (*ace-3*) and mosiac mapping of a synthetic lethal phenotype. *Neuron,* 1 : 165-173.

JOHNSON, C. D. & RUSSELL, R. L. (1975). A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. *Anal. Biochem.,* 64 : 229-238.

JOHNSON, C. D. & RUSSELL R. L. (1983). Multiple molecular forms of acetylcholinesterase in the nematode *Caenorhabditis elegans. J. Neurochem.,* 41 : 30-46.

KOLSON, D. L. (1984). *New acetylcholinesterase-deficient mutants and a new class of acetylcholinesterase in the nematode* Caenorhabditis elegans. Ph. D. Diss., Univ. Pittsburgh, PA, USA, 138 p.

KOLSON, D. L. & RUSSELL, R. L. (1985*a*). New acetylcholinesterase-deficient mutants of the nematode *Caenorhabditis elegans. J. Neurogenet.,* 2 : 69-91.

KOLSON, D. L. & RUSSELL, R. L. (1985*b*). A novel class of acetylcholinesterase, revealed by mutation, in the nematode *Caenorhabditis elegans. J. Neurogenet.,* 2 : 93-110.

MILLER, J. H. (1972). Purification of B-galactosidase. In : *Experiments in molecular genetics.* Cold Spring Harbor Laboratory, New York : 398-404.

PREMACHANDRAN, D., BACOU, F., BRIDE, J. M., & BERGÉ, J. B.