Purification of the enzyme acetylcholinesterase (AChE. 3.1.1.7) from *Meloidogyne incognita* and *Heterodera zeae*

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SUMMARY

Acetylcholinesterase (AChE. 3.1.1.7) enzyme from the juveniles (J2) and females of both *Heterodera zeae* and *Meloidogyne incognita* was purified by ammonium sulphate precipitation, Sephadex G-25, Sephadex G-200, DEAE-cellulose and DEAE-sephadex A-50 gel chromatography. Highly purified AChE preparations were obtained from both stages of the plant parasitic nematodes. The existence of the cholinergic system in nematodes is discussed, especially as the greater enzyme activity in females than in J2 suggests a correlation with development in the nematodes.

RÉSUMÉ

Purification de l'acétylcholinestérase (AChE, 3.1.1.7) provenant de Meloidogyne incognita *et* Heterodera zeae

L'acétylcholinestérase (AChE 3.1.1.7) provenant de juvéniles (J2) et de femelles de *Meloidogyne incognita* et *Heterodera zeae* est purifiée par précipitation à l'aide de sulfate d'ammonium et chromatographie sur gel utilisant Sephadex G-25, Sephadex G-200, DEAE-cellulose et DEAE-sephadex A-50. Des préparations hautement purifiées d'AChE ont ainsi été obtenues à partir de l'un et l'autre stades de ces deux nématodes phytoparasites. L'existence d'un système cholinergique chez les nématodes est discutée, l'activité enzymatique plus élevée chez les femelles que chez les J2 suggérant une corrélation avec le développement du nématode.

Acetylcholinesterase (acetylcholine hydrolase E.C. 3.1.1.7, AChE) is a very important enzyme in biological systems because of its vital role in nerve impulse transmission. Also, it is the target for organophosphate and carbamate pesticides (Nelmes, 1970; Wright & Awan, 1976). Rhode (1960) provided the first evidence for the presence of AChE in the plant parasitic nematodes, *Trichodorus christei, Pratylenchus penetrans, Xiphinema americanum* and *Helicotylenchus nannus*. Histochemical localization of cholinesterases within the neuromuscular axons of nematodes was reported by Lee (1962) and subsequent studies involving disc electrophoretic analysis of tissue homogenates of *Meloidogyne javanica* and *M. incognita* revealed the presence of a single strong band of AChE activity (Dickson, Huising & Sasser, 1971). The presence of specific AChE in the nerve ring of *Aphelenchus avenae* and some free living nematodes was demonstrated by Wright and Awan (1976) using specific histochemical method. More recently polyacrylamide gel slab electrophoretic analysis of several *Meloidogyne* spp. indicated the presence of AChE as a cathodal fraction which hydrolysed acetyl and butyrylthiocholine iodide (Esbenshade & Triantaphyllou, 1986). Thus, only limited qualitative information on AChE in plant nematodes is known, and an essential requirement for gaining quantitative data is a method for purifying this enzyme. This paper reports a protocol for the purification of AChE enzyme from two plant parasitic nematodes *M. incognita* and *Heterodera zeae*, which yielded highly purified AChE enzyme of use in subsequent studies on characterization of the enzyme.

Material and methods

CHEMICALS

The chemicals used were : acetylthiocholine iodide (AcThCh), 5,5' - dithiobis 2-nitrobenzoic acid (DTNB), eserine and bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, Missouri, USA); sephadex G-25, particle size 50-150 μ and sephadex G-200, particle size 10-40 μ and DEAE-sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden); DEAE-cellulose (diethyl aminoethyl-cellulose) (Whatman, U.K.) and polyacrylamide gel (Merck, West Germany). All the

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other reagents were of analytical grade and highest purity available commercially.

Nematodes

The root-knot nematode, *Meloidogyne incognita* originally obtained from a single eggmass was maintained on tomato *(Lycopersicon esculentum* cv. Pusa Ruby) to ensure the constant supply of juveniles (J2) and females. Likewise, the stock culture of corn cyst nematode (CCN), *Heterodera zeae* was cultured on corn (*Zea mays* cv. Ganga-5) by inoculating J2 emerged from a single cyst under greenhouse conditions and subcultured further.

Collection of nematodes and eggs

Second-stage juveniles (J2) : Standard procedures were followed to collect the J2 from eggmasses in case of *M. incognita* and from cysts in case of *H. zeae.* Freshly emerged J2 were collected and axenized by placing them in 500 ppm of streptomycin sulphate solution for 8 h as per procedure outlined by Dasgupta and Ganguly (1975), and J2 were pooled together. Batches of J2 in sterile double distilled water, concentrated by centrifugation (1500 *g* for 2 min at 4 °C) and decantation of supernatant, were quick frozen with dry ice, and stored at — 12 °C in sterile 8 × 75 mm vials upto 7 days without loss of any enzyme activity.

Females : Followed the method given by Dropkin, Smith and Myers (1960), for extracting females of *M. incognita* from galled roots of tomato, and young (white) females of *H. zeae* from the infested plant roots harvested 18-21 days after inoculation with J2 of *H. zeae.* The females, extracted from the roots were collected, washed and surface sterilized and stored via methods outlined in the preceding section for J2.

Eggs : Standard procedures were followed to collect the eggs of *M. incognita* and *H. zeae* and the eggs were surface sterilized and stored as per the procedure adopted in case of J2.

Preparation of crude extract

Four ml of J2 of *M. incognita* or J2 of *H. zeae* suspension was thawed and homogenized in 5 ml of 0.1 M sodium phosphate buffer, pH 7.6, by sonication with a vibronics 250 W ultrasonic tissue homogenizer model VP 4 P-2 at 25 000 cps for 120 seconds at 4 °C to obtain at least 90 % breakage. The homogenates thus obtained, were centrifuged at 4 °C for 5 min at 16 000 *g*. The clear supernatant was collected for assaying the AChE activity.

Two ml suspension of eggs of *M. incognita* or *H. zeae* was thawed and homogenized in 2 ml of 0.1 M sodium phosphate buffer, pH 7.6 and prepared the crude extract as mentioned above.

The tissue homogenates of females of *M. incognita* and *H. zeae* were prepared for detection of the enzyme by subjecting batches of nematodes, each one containing 3000 to 4000 nematodes in 3-4 ml of 0.1 M sodium phosphate buffer, pH 7.6, to an electrically operated teflon-glass Virtis type tissue homogenizer initially at lower speed of 3000 rpm for 30 s and for another 60 s at a higher speed of 14 000 rpm at 4 °C. Further, rupturing of tissue was accomplished through sonication at 25 000 cps for 30 s at 2-4 °C. Clear supernatant was obtained by refrigerated centrifugation at 14 000 *g* for 30 min for enzyme assay.

Enzyme assay

AChE activity was determined using the method of Ellman *et al.* (1961). The principle of this method is based on the production of thiocholine from acetylthiocholine by acetylcholinesterase. Thiocholine reacts with the reagent DTNB and a yellow anion of 5-thio-2-nitrobenzoic acid is produced which has maximum absorbance at 412 nm. The reaction mixture for routine assay consisted of 3.0 ml sodium phosphate buffer pH 8.0, 20.0 µl acetylthiocholine iodide substrate (0.075 M), 100.0 µl DTNB (0.01 M) and 50.0 µl enzyme.

One enzyme unit is defined as that amount of enzyme required to hydrolyse 1 µM of acetylthiocholine per min at 30 °C, 0.1 M sodium phosphate buffer, pH 8.0. Specific activity is expressed as enzyme units per mg of protein.

Determination of proteins

Protein concentration of the crude extracts and subsequent eluants were determined either by the method of Lowry *et al.* (1951) or by measurement of the extinction at 280 nm *vs* 260 nm using crystalline bovine serum albumin as standard (Layne, 1957).

Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the procedure of Davis (1964). The gel concentration for the separating gel was 7.2 % with 2.5 % cross-linking and the running solution was Tris-glycine buffer (pH 8.65, upper buffer 0.054 M; lower buffer 0.108 M). A stacking gel was not used. The applied current was 2 mA per tube for 5 h at 4 °C. Protein was stained with Coomassie Blue according to the method of Chambach *et al.* (1967). AChE activity was detected using acetylthiocholine iodide as per method of Karnovasky and Roots (1964) as modified by Tripathi and O'Brien (1977).

Purification procedure

Unless otherwise stated, all the operations of purification were carried out in cold room at 2-4 °C. Crude extract was prepared as above and purified as follows :

Step-1 : Initially, solubilization of the enzyme was

investigated by adding oxyethelene lauryl ether to homogenates to give 0.2 % solution and the mixture was allowed to sit at 4 °C for 24 h. Attempts were also made to homogenize the tissue in a solution containing 0.1 M sodium phosphate buffer, pH 7.6; 0.2 M NaCl; 0.5 % Triton X-100 and 0.001 M Na_2EDTA. Subsequently, it was observed that exclusion of oxyethelene lauryl ether or Triton X-100 and Na_2EDTA did not facilitate solubilization of the enzyme considerably. It was further observed that the enzyme activity increased when the homogenates were left standing in a cold room at 4 °C several hours or when they are frozen and thawed, suggesting that enzyme was being solubilized of activated from particulate fraction.

$(NH_4)_2SO_4$ saturation - Solid ammonium sulphate (enzyme grade) was added (17.6 g/100 ml) slowly with constant stirring to supernatant, collected from preceding step to obtain 30 % saturation. The solution was centrifuged immediately at 14 600 × *g* at 4 °C for 20 min. Ammonium Sulphate (27 g/100 ml) was added to the supernatant to bring 70 % saturation. This solution was stored at 4 °C for 3 h and then centrifuged at 14 600 *g* for 20 min to obtain the precipitate. The precipitate was dissolved in 3 ml of cold distilled water and then recentrifuged at 14 600 *g* at 4 °C for 20 min.

Step-2 : Sephadex G-25 gel chromatography - supernatant obtained from Step-1, having AChE activity was passed through a Sephadex G-25 gel column (15 × 1 cm) previously equilibrated with sodium phosphate buffer (0.1 M, pH 7.6). The enzyme was eluted with the same equilibration buffer and the elution rate was 0.6 ml per min. The fractions showing acetylcholinesterase activity were pooled together, concentrated through dialysis for 5 h at 4 °C for passing them to next step. And the concentrations of enzyme and protein in different samples, at this stage, ranged from 7.0 to 9.0 units/ml and 2-4 mg/ml of tissue homogenates, respectively.

Step-3 : Sephadex G-200 gel chromatography — The column was prepared as per manufacturers instructions. Sephadex G-200 gel column (15 × 1 cm) was equilibrated with at least five bed volumes of phosphate buffer (0.1 M, pH 7.6). The dialized enzyme solution obtained from Step-2 was applied on the top of the Sephadex G-200 column, eluted with the same buffer (equilibration buffer) and 15-20 fractions of 3 ml each were collected. The elution rate was 0.4 ml per minute. The fractions showing AChE activity were pooled together concentrated through sucrose dialysis (5 h) for passing them to the next step.

Step-4 : DEAE-cellulose chromatography — The dialysed enzyme solution obtained from Step-3 was applied onto an anion exchanger, DEAE-cellulose column (12 × 1 cm) which was equilibrated with phosphate buffer (0.01 M, pH 7.6). The column was eluted with 10 ml of phosphate buffer (0.01 M, pH 7.6) and then the column was eluted with buffer containing a linear gradient of NaCl from 0 to 1.0 M. The elution rate was 0.7 ml per minute. Fractions showing AChE activity were pooled together concentrated through sucrose dialysis for passing them to the next step.

Step-5 : DEAE-sephadex A-50 gel chromatography — The dialyzed enzyme solution obtained from Step-4 was added to a DEAE-sephadex A-50 gel column (10 × 1 cm) which was equilibrated with sodium phosphate buffer (0.1 M, pH 7.6) containing 0.5 M NaCl. The column was eluted with 10 ml of same buffer, and elution was continued using the same buffer with a higher salt concentration (1.0 M NaCl). The elution rate was 0.5 ml per minute. The fractions showing AChE activity were collected.

The purity of enzyme was checked through polyacrylamide gel-electrophoresis and staining gels for protein as well as enzyme activity.

Results

The crude preparations were observed to retain their activity without any loss when stored at — 12 °C for at least 4 weeks. Analysed on the basis of equivalent tissue homogenates (four replicates for each stage) it is more than apparent that the total enzyme activity was slightly more in females residing inside roots than those in freshly emerged J2 of both *M. incognita* and *H. zeae.* The specific activity of the enzyme was greater in females of both nematode species than in J2 (Table 1). A summary of the acetylcholinesterase purification from *H. zeae* and *M. incognita* are presented in Tables 2 and 3. Figures 1 and 2 represent typical elution profiles AChEs of *H. zeae* and *M. incognita* when run through sephadex G-200, DEAE-cellulose and DEAE-sephadex A-50.

Enzyme preparations from freshly emerged J2 of *H. zeae* obtained through sephadex G-200 column exhibited one major peak showing high AChE activity, and one minor peak (Fig. 1 A) showing low AChE activity. Subsequent fractionation through ion exchanger failed to separate the minor peak showing AChE activity (Fig. 1 B). Fractionation through DEAE-cellulose and DEAE-sephadex A-50 column resulted in an increase of sepecific activity of 230 and 357 respectively and purification to the order of 92 and 142 fold increases over crude extract respectively (Table 2).

Enzyme preparations from females of *H. zeae* obtained through Sephadex G-200 column exhibited two major peaks showing AChE activity (Fig. 1 D). Fractions under both the peaks were pooled together and passed through a DEAE-cellulose column. The elution profile (Fig. 1 E) again demonstrated the presence of two peaks, first and second peaks were obtained at the concentration of 0.3 M (0.20 to 0.38 M) and 0.8 M (0.75 to

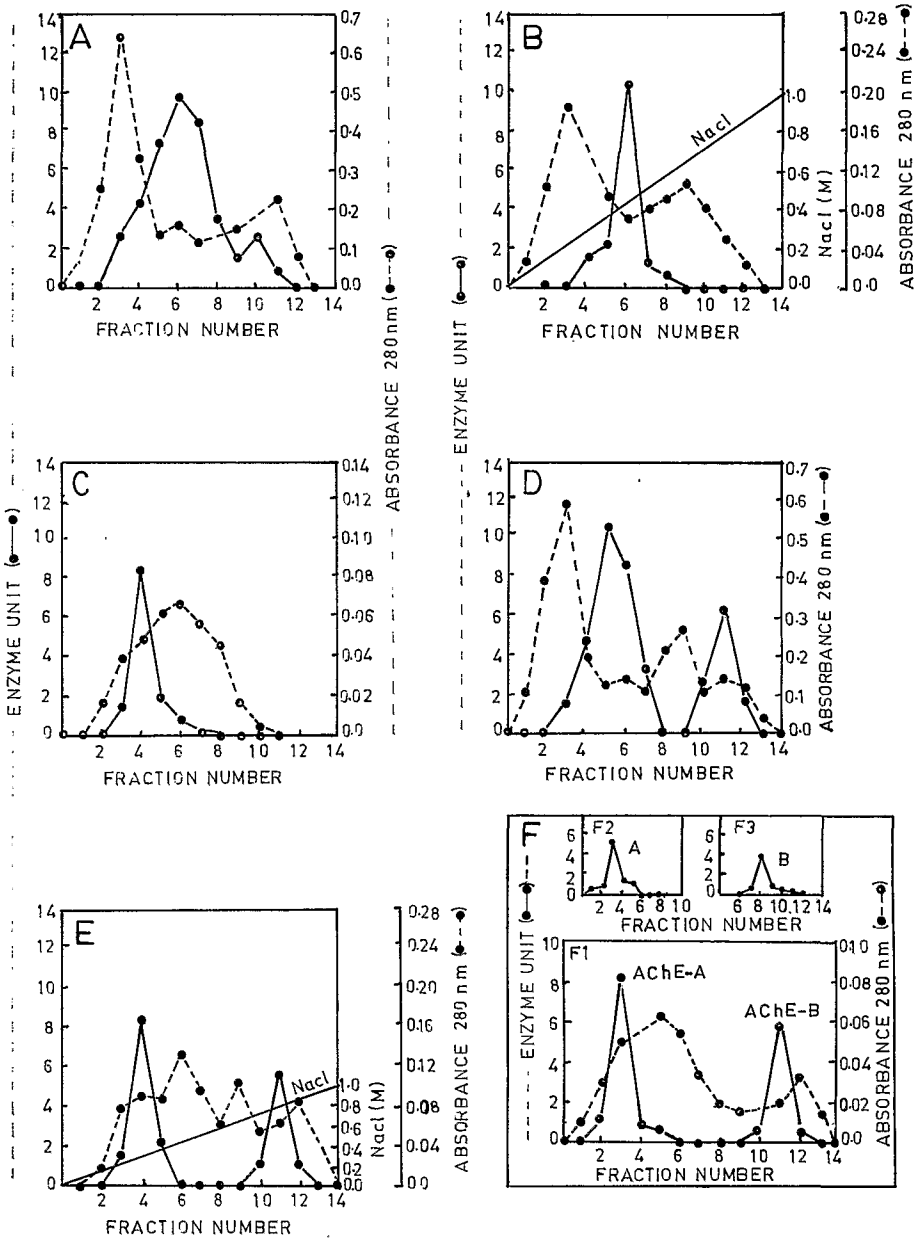

Fig. 1. Elution profiles of AChE from J2 of *H. zeae*, when passed through - A : Sephadex G-200 column; B : DEAE-cellulose column; C : DEAE-Sephadex A-50 column chromatography. Elution profiles of AChE from females of *H. zeae* when passed through - D : Sephadex G-200 column; E-DEAE-cellulose column; F_1 : DEAE-Sephadex A-50 column chromatography; F_2 : Rechromatography of AChE-A & F_3 : Rechromatography of AChE-B through DEAE-Sephadex A-50 gel column.

0.82 M) of NaCl respectively. These two peaks were designated as AChE-A and AChE-B respectively in correspondence with order of progressive elution pattern. Initially fractions under the peak A and B were pooled together and chromatographed on to a DEAE-Sephadex A-50 column and subsequently each peak rechromatographed separately on a second DEAE-sephadex A-50 column. The elution profile of separated peaks when rechromatographed are shown in Figs 1, 2, 3. After DEAE-Sephadex A-50 stage, purification to the

Table 1

AChE activity in crude extracts of *H. zeae* and *M. incognita*

Nematode	*Crude homogenate*	**Enzyme activity (units) (Mean & ± SE)*	***Protein (mg/ml) (Mean & ± SE)*	****Specific activity (Mean & ± SE)*
H. zeae	crude extract of J_2	7.25 ± 0.38	2.86 ± 0.09	2.53 ± 0.04
	crude extract of females	10.15 ± 0.46	3.48 ± 0.18	2.94 ± 0.05
M. incognita	crude extract of J_2	8.28 ± 0.23	4.25 ± 0.15	1.95 ± 0.06
	crude extract of females	10.40 ± 0.29	4.95 ± 0.17	2.10 ± 0.04

Each value is a mean of 4 replicates.

* Enzyme activity is expressed as units per ml of solution. Units are calculated on the basis of equivalent tissue homogenates. For definition of enzyme unit see the text.
** Protein is expressed as mg/ml of tissue homogenate.
*** Specific activity is expressed as enzyme unit/mg of protein.

Table 2

Summary of purification of AChE from J_2 and females of *H. zeae**

Steps	AChE from J2 of *H. zeae*					AChE from females of *H. zeae*				
	Protein (mg)	*Enzyme activity (units) ***	*Specific activity*	*Yield (%)*	*Purification fold*	*Protein (mg)*	*Enzyme activity (units) ***	*Specific activity*	*Yield (%)*	*Purification fold*
1. Crude	30.0	75.9	2.5	100.0	1.0	26.9	79.2	2.9	100.0	1.0
2. Ammonium sulphate fractionation 30-70 %	10.50	55.7	5.3	73.4	2.1	9.0	63.0	7.0	79.5	2.4
3. Sephadex G-25 gel filtration	7.5	51.0	6.8	67.2	2.7	6.0	53.8	8.9	67.9	3.1
4. Sephadex G-200 gel filtration	0.51	41.2	80.8	54.3	32.3	0.45	40.8	90.7	51.5	31.2
5. DEAE-cellulose chromatography	0.07	16.1	230.0	21.2	92.0	0.11	32.0	290.9	40.4	98.9
6. DEAE-sephadex A-50 chromatography	0.035	12.5	357.0	16.5	142.8	—	—	—	—	—
a. Isozyme-A	—	—	—	—	—	0.03	14.0	468.0	17.7	159.0
b. Isozyme-B	—	—	—	—	—	0.02	12.5	624.0	15.8	212.2

* Results represent an average of at least two independent runs.
** For definition of enzyme unit see the text.

order of 159 fold and 212 fold (Table 2) over crude extracts were observed for AChE-A and AChE-B (isozymes).

Enzyme preparation from freshly emerged J2 of *M. incognita* obtained through Sephadex G-200 column exhibited one major peak showing high AChE activity and two minor peaks showing low AChE activity (Fig. 2 A). Enzyme purified separately from minor peaks could not be differentiated as isozymes (Fig. 2 B). After DEAE-cellulose and DEAE-Sephadex A-50 stages, the

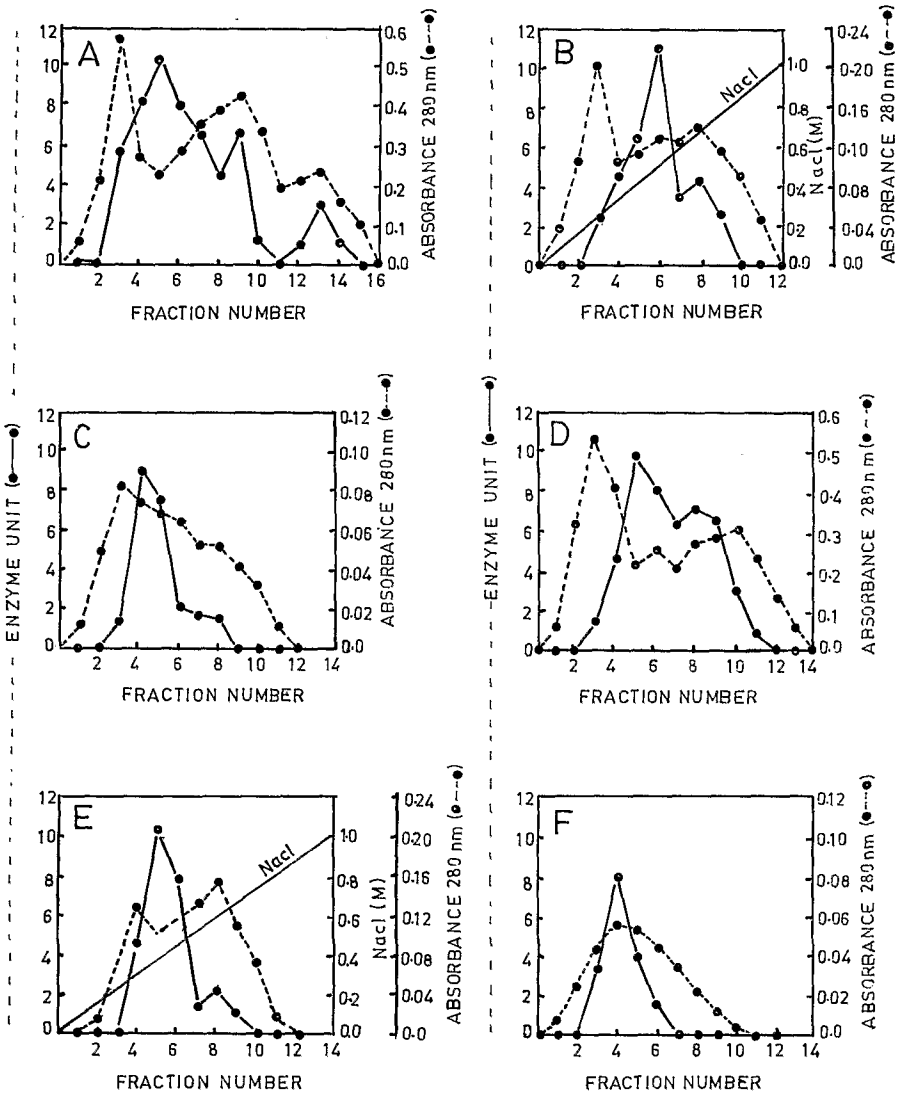

Fig. 2. Elution profiles of AChE from J2 of *M. incognita*, when passed through-A : Sephadex G-200 column; B : DEAE-cellulose column; C : DEAE-Sephadex A-50 column chromatography, elution profiles of AChE from females of *M. incognita*, WHEN PASSED THROUGH D : Sephadex G-200 column; E : DEAE-cellulose column and F : DEAE-Sephadex A-50 gel column chromatography.

increase in specific activities was 171- and 520-fold respectively, and purification to the order 274 fold after the last step of fractionation (Table 3).

The females of *M. incognita* obtained from roots also showed one major peak of AChE activity and one minor peak showing very little activity of AChE when eluted through Sephadex G-200 column (Fig. 2 D). Enzyme when eluted through DEAE-cellulose column gave one major peak of activity at the NaCl concentration of 0.32 to 0.54 M (Fig. 2 E). At DEAE-cellulose and DEAE-Sephadex A-50 stages the specific activities were 134 and 424 respectively with final purification to the order of 202 fold (Table 3).

No AChE activity could be detected from the eggs of *H. zeae* and *M. incognita*.

Discussion

Studies on AChE from plant parasitic nematodes so far, have been confined to qualitative aspects such as detection, histochemical localization of the enzyme, and electrophoretic analysis. However, most of the histo-

Table 3

Summary of purification of AChE from J2 and females of *M. incognita**

Steps	AChE from J2 of *M. incognita*					AChE from females of *M. incognita*				
	Protein (mg)	*Enzyme activity (units)* **	*Specific activity*	*Yield (%)*	*Purification fold*	*Protein (mg)*	*Enzyme activity (units)* **	*Specific activity*	*Yield (%)*	*Purification fold*
1. Crude	62.4	121.6	1.9	100.0	1.0	45.60	98.0	2.1	100.0	1.0
2. Ammonium sulphate fractionation 30-70 %	27.9	98.0	3.5	80.6	1.8	16.20	72.5	4.5	74.0	2.1
3. Sephadex G-25 gel filtration	14.7	86.8	5.9	71.4	3.0	10.45	65.8	6.3	67.1	3.0
4. Sephadex G-200 gel filtration	1.20	54.6	45.5	44.9	23.3	0.85	48.4	57.0	49.4	27.1
5. DEAE-cellulose chromatography	0.20	34.3	171.6	28.2	89.2	0.20	26.9	134.5	27.4	64.0
6. DEAE-sephadex A-50 chromatography	0.045	23.4	520.0	19.2	274.0	0.041	17.4	424.4	17.7	202.0

* Results represent an average of at least two independent runs.
** For definition of enzyme unit see the text.

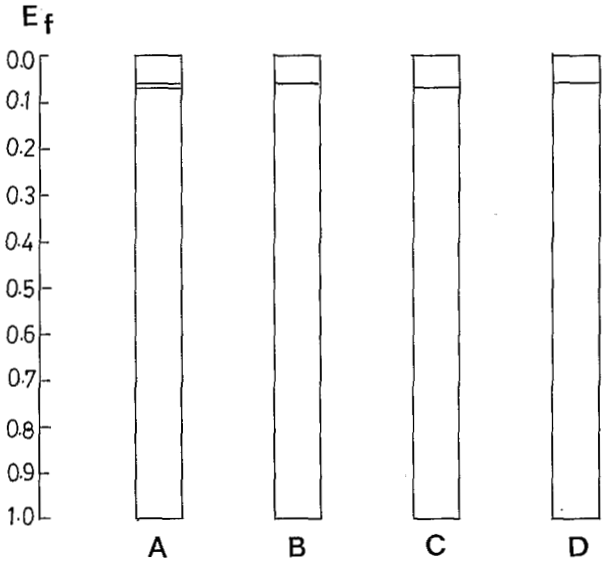

Fig. 3. ELECTROPHORETOGRAMS OF - A : Purified AChE enzyme of *H. zea* females; B : purified AChE-A isozyme of *H. zeae* females; C : purified AChE-B isozyme of *H. zeae* females; D : purified AChE enzyme of J_2 *H. zeae*.

chemical studies were not sufficiently specific to separate ChE and AChE activity. Studies on AChE conducted by Rohde (1960), Dickson, Huising and Sasser (1971), and Wright and Awan (1976) dealt with qualitative aspects. Only recently, Esbenshade and Triantaphyllou (1985) demonstrated AChE of *Meloidogyne* spp. as a cathodal fraction in polyacrylamide gel slab electrophoresis, but their investigation did not deal with the purified enzyme preparations which are essential for understanding kinetic and molecular properties of the enzyme. Using the given protocol, a highly purified AChE enzyme preparation was obtained from both the plant parasitic nematodes. These purified preparations were characterized by rapid hydrolysis of acetylthiocholine, inhibition at lower concentration of eserine (10^{-5} M). We recorded complete inhibition (100 %) of the purified enzyme after an exposure of 30 minutes to the inhibitor at molar concentrations of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. These observations coupled with data on enzyme kinetics which will be reported in a separate paper, prompt us to suggest the possible existence of a well developed AChE activity and possible cholinergic system in these nematodes. Nematodes have been demonstrated to possess an acetylcholinesterase system (Rohde, 1960; Benton & Myers, 1967; Johnson & Russel, 1983). AChE activity has been demonstrated in all nematode species in which it has been assayed (Johnson & Russel, 1983). All of the cholinesterase activity associated with the nerve ring has been shown to be due to specific AChE (Wright & Awan, 1976). Hence, Willet (1980) reports that there can be no doubt about the importance and generality of cholinergic neurons in the

nervous system of nematodes. Further studies on AChE, localization of choline acetyl transferase (CAT) in the ventral dorsal commissures of dorsal excitory motorneurons and establishment of use of acetylcholine by the excitory motorneuron in *Ascaris lumbricoides* (Johnson & Stretton, 1977; Johnson & Russel, 1983) indicate the cholinergic nature of central nervous system as well as neuromuscular transmission in nematodes. Our studies though limited to two nematode species and two life stages, indicate the differences in AChE activities between life stages of the same species on the one hand and remarkable differences in the specific activity of the enzyme on the other. In view of lack of sufficient data it is rather problematic to assess and reason the significance of such observations in terms of neurobiology, especially the cholinergic aspect of nematode nervous system. We did not observe any measurable AChE activity in the eggs of these nematodes, an experience shared previously by Dickson, Huising and Sasser (1971). This and our observations revealing the higher levels of enzyme activity in the adult females than in J2 of both nematode species suggests a correlation between development of the nervous system and increased cholinergic activity in nematodes. Sanderson (1969) also reported that AChE levels show an age-dependent increase in *Nippostrongylus brasiliensis.*

The existence of multimolecular forms of AChE in *Caenorhabditis elegans* have been reported (Johnson & Russel, 1983), but the record of AChE isozymes in *H. zeae* appears to be the first.

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