Proteolytic activity in *Caenorhabditis elegans*: soluble and insoluble fractions

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Abstract

Proteolytic activities of soluble and insoluble fractions of the free-living soil nematode *Caenorhabditis elegans* were measured across a range of substrates, temperatures and pH conditions. Several protease inhibitors were also tested under these conditions. Results of these studies indicate that proteolytic activity is present in cytosolic (CF) and non-cytosolic (NCF) fractions of *C. elegans* extracts at every condition of pH, temperature and buffer assayed. On the other hand, the use of different protease inhibitors demonstrated the existence of exo and endoproteases types in CF as well as NCF. Moreover our results show that the use of two protease inhibitor mixed types proposed by several authors are not enough to avoid this lytic activity when homogenates of this nematode are employed in biochemical assays.

Introduction

The free-living nematode *Caenorhabditis elegans* is one of the most widely used experimental models in modern biology. This nematode can be easily mantained in culture and it is easy to propagate large numbers for use in biochemical studies. It has also been employed in genetic, neuroanatomical or behavioural studies (Ward, 1988), as well as in comparative studies with parasitic helminths (Lackey *et al*., 1989; Cox *et al*., 1990; Kingston & Petitt, 1990; Richer *et al*., 1992; Cross & Barry, 1995).

A major problem that often confounds biochemical studies on nematodes is the degradation of proteins and the fact that there is little information available about inhibitors that can be used to prevent proteolysis in nematode tissue preparations (Rohrer *et al*., 1990; Schaeffer & Haines, 1990). Therefore, the aim of this study was to characterize the proteolytic activities in nematode tissue with respect to pH, temperature and inhibitor sensitives using samples that are frequently used in biochemical assays: i.e. non-cytosolic (NCF) and cytosolic fractions (CF). These findings are discussed in terms of their implications in biochemical assays.

Materials and methods

Collection of Caenorhabditis elegans *adults and larvae*

Adults and larvae of the free-living soil nematode *C. elegans* wild type (WTN_2) were employed. Asynchronous cultures with respect to age and developmental stage were maintained on agarose using 100 mm petri dishes containing cholesterol, previously seeded with the *Escherichia coli* OP50 strain, and incubated at 22°C for 3–5 days (Brenner, 1974) with slight modifications. When the *E. coli* food source appeared by inspection to have been nearly exhausted, the nematodes were collected by rinsing the plates extensively with M9 buffer. Suspensions were centrifuged briefly at 460 *g*, and the supernatant removed. Worms were used inmediately or stored at –80°C until use.

Preparation of worm extracts

Worm suspensions homogenized in a glass Potter-Elvejem (POBEL, Spain) were kept on ice throughout this procedure and homogenization was regularly

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interrupted to allow cooling. Microscopic examination revealed that the worms were totally broken open. The homogenized tissue was ultracentrifuged for 15 min at 60000 *g* at 4°C in a Beckman XL ultracentrifuge. The pellet (P_1) collected following centrifugation, was considered to be the non-cytosolic fraction (NCF). The supernatant was then centrifuged 30 min at 100000 *g*, and the resulting second supernatant was considered to be the cytosolic fraction (CF). The supernatant recovered was examined using a microscope to ensure that no larvae or shed cuticles had been transferred. In both preparations, protein measurement was performed inmediately using bovine serum albumin (BSA) as standard (Lowry *et al*., 1951; Peterson, 1983). The concentration of each sample was adjusted to 6 mg ml^{-1}, with distilled water or with buffers commonly used in biochemical assays (0.095 mM Mops pH 7.2, 50 mM Hepes pH 7.4 and 20 mM Tris pH 7.4), used immediately or frozen at –80°C until use.

Substrate gel electrophoresis

Electrophoretic analysis of the NCF and CF samples (30 μg protein diluted 3:1 in glycerol were applied per lane) was performed in a polyacrylamide gel electrophoresis, copolymerized with 0.15% w/v gelatin as substrate (Armas-Serra *et al*., 1995). Gels were electrophoresed for 2–3 h at 70 v per gel in a pH 8.3 Tris–glycine running buffer. Boiling and reducing agents were not used. Following electrophoresis, gels were incubated for 24 h at different temperatures (4°, 22° and 37°C) and pH conditions to allow the nematode products to act on the gelatin substrate. The buffers used were: 0.1 M citrate pH 3 and 5; 0.1 M Tris pH 7 and 8 with or without 2 mM $CaCl_2$ in overnight incubations. These conditions were selected to reflect the temperatures and pH values typically used in biochemical assays (4° and 37°C) as well as the optimal conditions for the biology of *C. elegans* (22°C). Following incubation, gels were stained with 0.1% Coomassie brilliant blue in 30% methanol and 10% acetic acid, and destained with the same solvent, until proteases appeared as clear bands on the blue background.

Azocoll assay

The chromogenic non-specific protein substrate azo-coupled hide powder (Azocoll 5 mg ml^{-1}) (Sigma Chemical Co, St. Louis, Missouri, USA) was assayed to detect proteolytic activity (Criado-Fornelio *et al*., 1992). The effect of pH and temperature on the proteolytic activity was tested over the pH range 3–8, and temperatures of 4°, 22° and 37°C. The following buffers were used: 0.1 M citric acid/sodium citrate pH 3 and 5 and 0.1 M PBS pH 7 and 8. Tubes containig 250 μl azocoll, 200 μl buffer and 50 μl sample (NCF or CF) were incubated for 24 h and then centrifuged for 5 min at 4700 *g*. Absorbance of released dye in the supernatant fractions was measured spectrophotometrically at 540 nm. Assays were performed in quadruplicate, and data calculated following subtraction of reagent blanks containing no sample.

Proteinase inhibitors

To determine which classes of proteases are found in NCF and CF, proteolytic activity was measured in the presence of several inhibitors at neutral pH, and different temperatures (4°, 22° and 37°C). These inhibitors were: 0.5 μg ml^{-1}, 1 μM and 5 mM leupeptin, 17 μg ml^{-1}, 200 μM and 5 mM phenylmethylsulphonylfluoride (PMSF), 5 mM N-Ó-p-tosyl-l-lysine-chloromethylketone (TLCK), 50 μg ml^{-1} soybean trypsin inhibitor (STI), 5 μM trans-epoxysuccinil (E-64), 0.7 μg ml^{-1}, 2 μM pepstatin A, 100 μM, 0.5 mM and 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM 1-10 phenantrolin and the exopeptidase inhibitor 10 mM amastatin. We also employed several inhibitors mixed, called mixed type one (MI) consisting of 0.5 mM EDTA, 0.5 μg ml^{-1} leupeptin, 0.7 μg ml^{-1} pepstatin A and 17 μg ml^{-1} PMSF (Rohrer *et al*., 1990) and mixed type two (MII) consisting of 100 μM EDTA, 1 μM leupeptin, 1 μM pepstatin A and 200 μM PMSF (Boheringer-Manheim Biochemical). Each of the inhibitors was diluted in water except PMSF, TLCK and pepstatin A which were in DMSO; these volumes were corrected for activity using DMSO blanks. Each of the inhibitors was assayed at all three temperatures.

Inhibition of proteolytic activity was also determined by incubating NCF and CF with each of the above inhibitors in the azocoll assays described above (Criado–Fornelio *et al*., 1992). The effects of each inhibitor were calculated by comparison to controls containing buffer and solvent, but no inhibitor. Each inhibitor was also corrected by subtracting a blank containing substrate, inhibitor, but water in place of homogenates of NCF or CF.

Results and discussion

The presence of carboxyl and thiol proteases in *C. elegans* is well documented (Sarkis *et al*., 1988). Results of the present study confirm the existence of these and also demonstrate the existence of metallo and serine

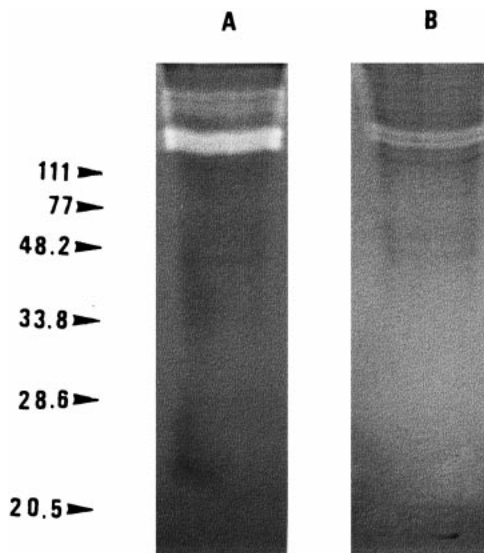

Fig. 1. Electrophoretic gelatin-profiles of *Caenorhabditis elegans* proteolytic activity. A, cytosolic fractions; B, non-cytosolic fractions.

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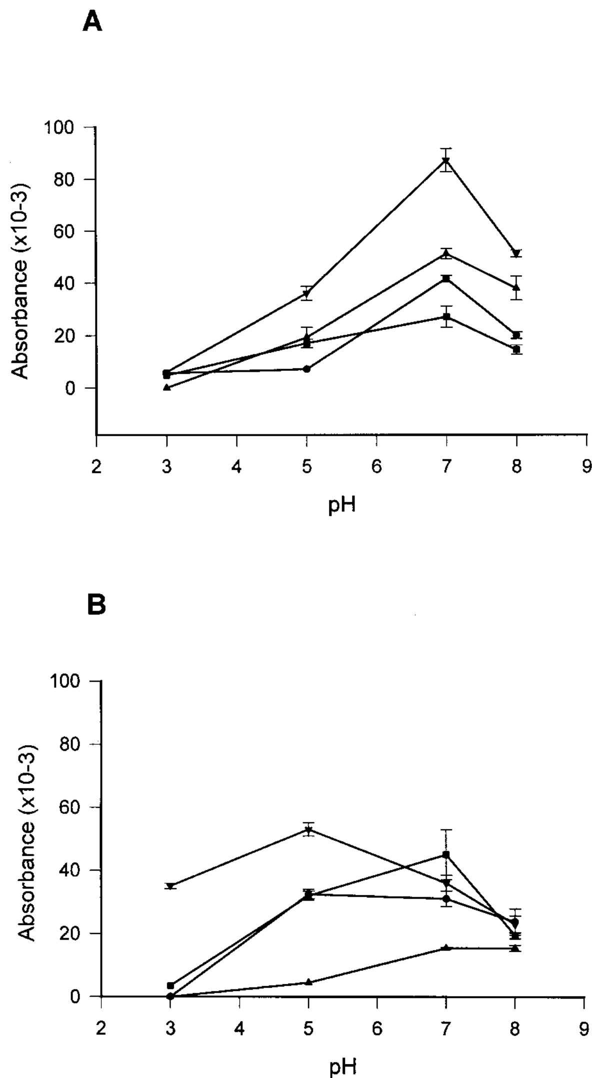

Fig. 2. pH values of *Caenorhabditis elegans* proteolytic activity when samples are diluted with distilled water (●) and with 0.095 mM MOPS (■), 20 mM TRIS (▲) and 50 mM HEPES (▼) at growth temperature (22°C). A, cytosolic fractions; B, non-cytosolic fractions.

protease activity in both CF and NCF of *C. elegans*. These enzyme activities were detected across a broad range of temperatures and pH conditions. Proteolytic activity was also tested using gelatin–substrate gels of non-reduced samples following 24 h incubation; results of these analyses are shown in fig. 1.

Results obtained in the azocoll assays are shown in fig. 2. In these studies, proteolysis of CF components ocurred across a broad pH range with peak azocoll hydrolysis ocurring at neutral pH. Proteolytic activities in NCF differed slightly from those in CF. Profiles depended on the buffer used to dilute the sample. We observed a significant decrease in proteolytic activity at pH 3, except when Hepes buffer was used in NCF.

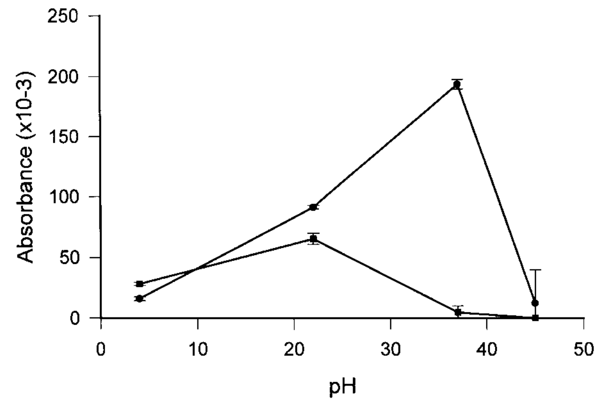

Fig. 3. Effects of temperature on *Caenorhabditis elegans* cytosolic (●) and non-cytosolic (■) fraction proteolytic activity.

Proteolytic activity in both NCF and CF appeared at all temperatures typically used in biochemical assays (fig. 3).

We measured a battery of class-specific proteinase inhibitors on azocoll degradation to determine classes of enzymes present in *C. elegans* extracts. The effects of these inhibitors on *C. elegans* protease activities was highly dependent on temperature and the fractions used in the assay (table 1).

Total inhibition of protease activity in CF or NCF did not occur with either of the inhibitor cocktails (Mixed I and II) tested (table 1). Indeed, inhibition was higher when protease inhibitors were tested separately. This was not surprising because: (i) in a mixture, low concentrations of inhibitors are used to avoid interference with experimental samples, and concentrations employed may be insufficient to inhibit proteolytic activity found in the homogenates; and (ii) crude extracts contain a mixture of proteases of differing classes and it is possible that inhibition of one in a mixture can often result in apparent activation of another (Salvesen & Nagase, 1989).

In conclusion, the present study provides new knowledge on proteolytic activities present in homogenates of *C. elegans*. We propose the use of 5 mM TLCK at 4°C when NCF are used, 5 mM 1-10 phenantrolin when NCF samples are tested at 22°C, and 5 mM PMSF or 5 mM TLCK when CF is tested at 22°C and 5 mM TLCK when CF is tested at 37°C. At 4°C, proteolytic activity in CF was extremely low and it may not be neccesary to employ inhibitors. This is also true when NCF samples are tested at 37°C. In any case, as Epstein & Liu (1995) proposed for each application specific inhibitors may have to be added or deleted, or their concentrations increased or decreased and these findings may be useful when *C. elegans* homogenates are used in biochemical assays.

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Table 1. Effect of inhibitors on protease activities from *Caenorhabditis elegans* non-cytosolic and cytosolic fractions at 4°, 22° and 37°C.

Protease inhibitors		% Inhibition				
		4°C		22°C		37°C
		NCF (X ± S.E.M.)	CF (X ± S.E.M.)	NCF (X ± S.E.M.)	CF (X ± S.E.M.)	CF (X ± S.E.M.)
Serine/cysteine protease	Leupeptin					
	0.5 μg ml^{-1}	15.58 ± 0.003	6.25 ± 0.008	0	0	0
	5 mM	9 ± 0.009	0	0	40 ± 0.007	0
	1 μM	62.61 ± 0.005	0	0	0	21.73 ± 0.001
	PMSF					
	17 μg ml^{-1}	17.24 ± 0.002	0	0	0	10.46 ± 0.004
	200 μM	72.41 ± 0.005	0	0	0	0
	5 mM	28.8 ± 0.009	0	0	100	0
	TLCK					
	5 mM	100	0	79.26 ± 0.006	100	82.55 ± 0.001
Serine proteases	STI					
	50 μ ml^{-1}	79.16 ± 0.001	0	4.47 ± 0.006	0	6.97 ± 0.023
Cysteine proteases	E-64					
	5 μM	64.13 ± 0.003	0	0	1.88 ± 0.019	62.79 ± 0.004
Aspartic proteases	Pepstatin A					
	0.7 μg ml^{-1}	3.125 ± 0.009	0	0	0	30.23 ± 0.002
	2 μM	14.67 ± 0.008	8.57 ± 0.008	0	0	0
Metallo proteases	EDTA					
	100 μM	3.125 ± 0.003	0	32.85 ± 0.005	0	0
	0.5 mM	15 ± 0.002	0	47.27 ± 0.009	0	0
	5 mM	32.52 ± 0.004	3.125 ± 0.002	63.51 ± 0.006	42.5 ± 0.004	0
	1-10 Phenantrolin					
	5 mM	0	0	89.09 ± 0.001	58.66 ± 0.002	13.04 ± 0.007
Aminopetidases	Amastatin					
	10 mM	65.51 ± 0.001	0	6.62 ± 0.007	0	11.59 ± 0.009
Mixed	Type 1	32.5 ± 0.006	0	9.8 ± 0.006	0	0
	Type 2	10 ± 0.002	0	0	0	15 ± 0.004

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