Effects of Hydrolytic Enzymes on Plant-parasitic Nematodes

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Abstract: Proteases, lipase, and chitinase killed *Tylenchorhynchus dubius in vitro* and in soil. *Tylenchorhynchus dubius* was more susceptible to the enzymes than *Pratylenchus penetrans*. Papain was the most effective protease, and other enzymes were less effective. Heating enzymes to 80 C for 10 min greatly reduced nematicidal effectiveness. Scanning electron micrographs showed that papain and chitinase produced structural changes in the cuticle of *T. dubius*. Lipase removed a thin outer layer. Papain removed material filling the striata, or furrow, between the horizontal bands. When added to soil, chitinase, lipase, collagenase, and proteases (papain and bromelain) decreased motility of *T. dubius* populations up to 75%. Bromelain was the most active in soil against *T. dubius*, and collagenase was the most active in soil against *P. penetrans*. *Key Words: Tylenchorhynchus dubius, Pratylenchus penetrans,* chitinase, papain, collagenase, lipase, cuticle, soil, scanning electron microscope.

Organic matter added to soil decreases populations of plant-parasitic nematodes (6, 9, 10). Nematodes are presumably exposed to different types of enzymes released during the decomposition of the different types of organic matter in soil. In this paper, we report the effects of several enzymes from plant tissues on plant-parasitic nematodes. The enzymes were used singly or in combination, *in vitro* and in soil. The influence of papain and collagenase on nematodes parasitic on animals has already been reported (1, 2).

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MATERIALS AND METHODS

Tylenchorhynchus dubius was extracted by sugar flotation and a tissue technique

(7) from a bluegrass turf rhizosphere. *Pratylenchus penetrans* was extracted from potato rhizosphere by sugar flotation.

Enzymes tested were the following commercial preparations: wheat-germ lipase, steapsin (lipase), and erepsin (protease) from Nutritional Biochemicals Corporation; collagenase and crystalline papain from Sigma Biochemicals; chitinase and pronase (protease) from Calbiochem; and bromelain from Dole Division of Castle and Cooke. In the first test, enzymes were dissolved in deionized water at 0.8 mg/ml. Two ml of a standardized nematode suspension containing 84 *T. dubius*/ml were mixed with 2 ml of enzyme solution in small plastic cups at room temperature (22 C). Treatments were replicated four times and tests were repeated twice. Live, motile nematodes were counted after 18, 36, and 48 h. This procedure was used in the succeeding tests.

In additional tests, as in the previous tests, nematode suspensions were combined with enzyme solutions to give concentrations of 0.01, 0.1, and 1 mg/ml of papain and wheat-germ lipase and 0.001, 0.01, and 0.1 mg/ml of chitinase in 0.05 N potassium phosphate buffer at pH 6.8. Motility counts were made after 24 and 48 h. For a control, enzyme preparations in double strength buffer were used so that, when they were added to an equal volume of nematode suspension after they were cooled, they would have the same enzyme concentration as the unheated nematode suspension. They were heated at 80 C for 10 min and cooled prior to being added to nematodes. All treatments were replicated 4 times, and the test was repeated twice.

To determine whether the enzymes were killing or merely immobilizing *T. dubius*, 1 ml of a nematode suspension, which was obtained from a bluegrass sod and contained 79 *T. dubius*, 25 *Hoplolaimus tylenchiformis*, and 5 *P. penetrans*, was mixed with enzyme solutions in water to give concentrations of 1 mg of papain, wheat-germ lipase, and collagenase, and 0.1 mg of chitinase/ml. After being incubated for 48 h at 22 C, the active nematodes were counted. Then the nematodes and enzymes were washed onto a 38-μm sieve and rinsed for 3 min with water to wash away the enzymes. Then the nematodes were washed from the sieve into glass petri dishes and motile ones were counted after 24 and 96 h.

Activity of papain, collagenase, lipase and chitinase (0.4 mg/ml) was tested in 0.1 N potassium phosphate buffer to determine influence of pH on enzyme activity against nematodes. After the mixtures were incubated for 16 h at 22 C, counts of motile nematodes were made.

The effects of a protease inhibitor, pentachloronitrobenzene (PCNB) (3), on papain activity against *T. dubius* was tested by adding 0.2 mg of PCNB (a.i.)/ml with and without 0.4 mg of papain/ml. Counts were made after 24 h.

To determine whether morphological changes, visible with a light microscope, occurred during immersion in enzymes, *T. dubius* was incubated for 16 h at 22 C in chitinase, 0.1 mg/ml of deionized water, or water alone, and then stained in iodide-potassium iodide in sulfuric acid (4). *Tylenchorhynchus dubius* was also incubated for 16 h at 22 C in a similar manner in 1 mg of wheat-germ lipase/ml of water, and then stained for lipids after the method of Glick (4).

The influence of these enzymes on the cuticle of *T. dubius* was determined with the scanning electron microscope. After *T. dubius* was incubated for 2.5 h at 22 C in 0.1 N potassium phosphate buffer (pH 6.8) containing 1 mg of lipase or papain or 0.1 mg of chitinase/ml, the nematodes were pelleted by centrifugation at 1,000 *g* for 2 min. They were re-suspended in 35% (w/vol.) sugar solution, centrifuged at 1,000 *g* for 2 min again, and then removed with a pipette from the top of the solution. The nematodes were placed on a 0.22-mu millipore filter under vacuum to remove the sucrose solution and washed repeatedly with deionized water. Nematodes on the membrane filters were immediately frozen at −50 C for 2 h and vacuum-dried. Samples were stored over dessicant at −5 C. Control nematodes were handled in the same manner but without enzymes. Sections of each filter disc containing approximately 15-30 nematodes were mounted with graphite DAG (Acheson Colloid Co.), shadow-cast with gold in 360° rotation, and viewed at 5,000 X with a scanning electron microscope (ETEC Corp. California, Autoscan SEM). The first five nematodes with clearly visible

surface features were photographed at mid-section.

Soil infested with *P. penetrans* and *T. dubius* was mixed with either papain, chitinase, collagenase, wheat-germ lipase, or bromelain at rates of 4 and 40 mg/kg. Treatments were replicated 4 times and tests were repeated twice. After 3 weeks, nematodes were extracted from 100 gm of soil by sugar flotation and motile nematodes were counted.

RESULTS

In nonbuffered solutions, papain, chitinase, and wheat-germ lipase killed all *T. dubius* in 48 h (Table 1). Although not given in Table 1, *P. penetrans* and *H. tylenchiformis* were more resistant than *T. dubius* to papain; 37% of *P. penetrans* and 28% of *H. tylenchiformis* were still motile after 48 h while *T. dubius* was completely immotile.

Toxicity of papain and lipase against *T. dubius* decreased with dilution of the enzyme, but chitinase was equally toxic at high and low concentrations (Table 2). Heating destroyed activity of chitinase and lipase and most of the activity of papain for 24 h, but chitinase and lipase solutions showed some activity after 48 h.

After 24 h, all *T. dubius* in collagenase were immobile and over 80% of those in papain and chitinase were also. As in the previous tests, lipase was less effective, immobilizing only 57% of *T. dubius*. *H. tylenchiformis* and *P. penetrans*, as well as the microbivorous nematodes, were barely affected by the enzymes. After the enzymes were washed away, *T. dubius* was still immotile after 96 h in collagenase, papain, and chitinase. Of those which had been treated with lipase, only 41% were immotile; thus a few regained motility. *H. tylenchiformis*, *P. penetrans*, and the microbivorous nematodes, appearing unaffected by the enzymes, were still motile and active.

Acidity had a great influence on enzyme activity (Table 3). Collagenase and papain were more active at pH 6 than at pH 5. Papain was ineffective at pH 5. Chitinase and lipase were effective at pH 5 and pH 6, and also reduced the motility of *P. penetrans* 20-30% at pH 5 and 6.

PCNB reduced effectiveness of papain against *T. dubius*. Papain reduced motility of *T. dubius* 77%; PCNB alone reduced it 27%; but PCNB plus papain reduced it

TABLE 1. Influence of some hydrolytic enzymes on motility of *Tylenchorhynchus dubius in vitro* after 18, 36, and 48 h.

Enzyme (0.4 mg/ml)	Percent of nematodes motile		
	18 h	36 h	48 h
Water alone	92 a[y]	85 a	88 a
Wheat-germ lipase	86 a	31 bc	0 d
Papain	25 c	8 d	9 d
Chitinase	67 b	48 b	0 d
Collagenase	82 c	46 b	22 c
Steapsin	80 a	55 b	53 b
Pronase	90 a	99 a	53 b
Promelain	88 a	88 a	88 a
Erepsin	89 a	87 a	72 a
Mixture of all enzymes[z]	53 b	25 c	23 c

[y]Figures followed by same letter not significantly different from each other, according to Duncan's Multiple Range Test ($P=0.05$).
[z]All seven enzymes were each used at 0.4 mg/ml in mixture and gave a total enzyme content of 2.8 mg/ml.

TABLE 2. Effects of concentration and heat inactivation on toxicity of enzymes to *Tylenchorhynchus dubius in vitro*.

Enzyme[x]	Concentration (mg/ml)	Percent of nematode motile[y]	
		24 h	48 h
None	—	100 a	95 a
Chitinase	0.001	78 b	38 c
	0.01	74 b	35 c
	0.1	81 ab	35 c
	0.01 heated[z]	100 a	77 ab
Papain	0.01	96 a	65 b
	0.1	85 ab	19 c
	1.0	0 e	0 d
	1 heated	78 b	77 ab
Lipase (wheat germ)	0.01	78 b	54 b
	0.1	56 c	31 c
	1.0	24 d	11 d
	0.1 heated	0 e	85 a

[x]In 0.1 N potassium phosphate buffer at pH 6.8.
[y]Figures followed by same letters not significantly different from each other by Duncan's Multiple Range Test ($P=0.05$).
[z]Enzyme preparation heated at 80 C for 10 min; then cooled to room temperature before being mixed with nematodes.

TABLE 3. Effects of pH on toxicity of four hydrolytic enzymes to *Tylenchorhynchus dubius in vitro*.

Enzyme[y]	pH	Percent of motile nematodes
Buffer alone	5	94 a
	6	73 ab
Papain	5	91 a
	6	36 cd
Collagenase	5	40 c
	6	12 d
Chitinase	5	25 d
	6	39 c
Lipase	5	39 c
	6	46 c

[y]In 0.1 N potassium phosphate buffer for 16 h at 22 C.
[z]Each figure average of four replicates with 33 nematodes added/replicate. Figures followed by same letter not significantly different from each other, according to Duncan's New Multiple Range Test ($P=0.05$).

TABLE 4. Toxicity of four hydrolytic enzymes to *Pratylenchus penetrans* and *Tylenchorhynchus dubius* populations in soil.

Treatment	Concentration (mg/kg of soil)	No. of motile nematodes/100 gm soil[y]	
		P. petetrans	*T. dubius*
Water check	—	45 b	40 a
Papain	10	51 b	21 c
	40	55 b	19 c
	160	37 c	14 d
	160 heated[z]	91 a	47 a
Chitinase	4	56 b	18 c
Collagenase	4	72 a	47 a
	40	23 d	23 c
Lipase	4	38 c	21 c
Bromelain	40	38 c	9 d

[y]Average of four replicates 3 weeks after addition of buffered enzymes. Each figure followed by same letter not significantly different from each other, according to Duncan's Multiple Range Test ($P=0.05$).
[z]Enzyme heated to 80 C for 10 min.

only 24%, a reduction equal to that of PCNB alone.

The toxicity of enzymes in soil did not parallel their toxicity in aqueous solutions (Table 4). Papain was the most toxic in aqueous solutions against *T. dubius;* bromelain was the most toxic in the soil since 40 mg/kg of soil reduced *T. dubius* populations 76%. Papain, collagenase, lipase, and chitinase were moderately toxic in soil at one or more concentrations. *Pratylenchus penetrans* was not affected by papain in soil, and was affected most by 40 mg/kg of collagenase in soil.

There were no visible structural changes in the nematodes when they were examined with the light microscope.

Incubating *T. dubius* in active enzyme solutions for 2.5 h produced changes in the cuticle that were observed with the scanning electron microscope. The cuticle of the nontreated nematode (Fig. 1-A) shows regular, transverse striations or bands that are slightly indented and apparently rigid. The entire nematode appears to be coated with a very thin, slightly electron-opaque, layer. Chitinase removed the tops of the bands and caused lateral folding of *T. dubius* and loss of transverse rigidity (Fig. 1-B). Papain deepened striations and made them appear as deeper furrows (Fig. 1-C). Lipase appeared to remove the thin outer layer, mentioned before, on the untreated nematode, but did not change the appearance of the nematode cuticle enough to show in photographs. Lipase also caused some loss of rigidity of the transverse bands.

Chitinase and papain treatments for 2.5 h severely distorted many of the nematodes, perhaps as many as 80%, but micrographs were not made of severely distorted nematodes.

DISCUSSION

The data presented in this paper indicate that solutions of lipase, protease, and chitinase hydrolytic enzymes are toxic to nematodes but are more toxic to *T. dubius* than *P. penetrans* or *H. tylenchiformis*. Enzyme activity resulted in modification of the nematodes cuticle, an occurrence which was concomitant with death.

These results show that the enzymes actually killed, rather than temporarily immobilized *T. dubius* and thus were nematicidal. They were able to cause death only in *T. dubius,* however, *H. tylenchiformis* and *P. penetrans* were much more resistant to enzymes.

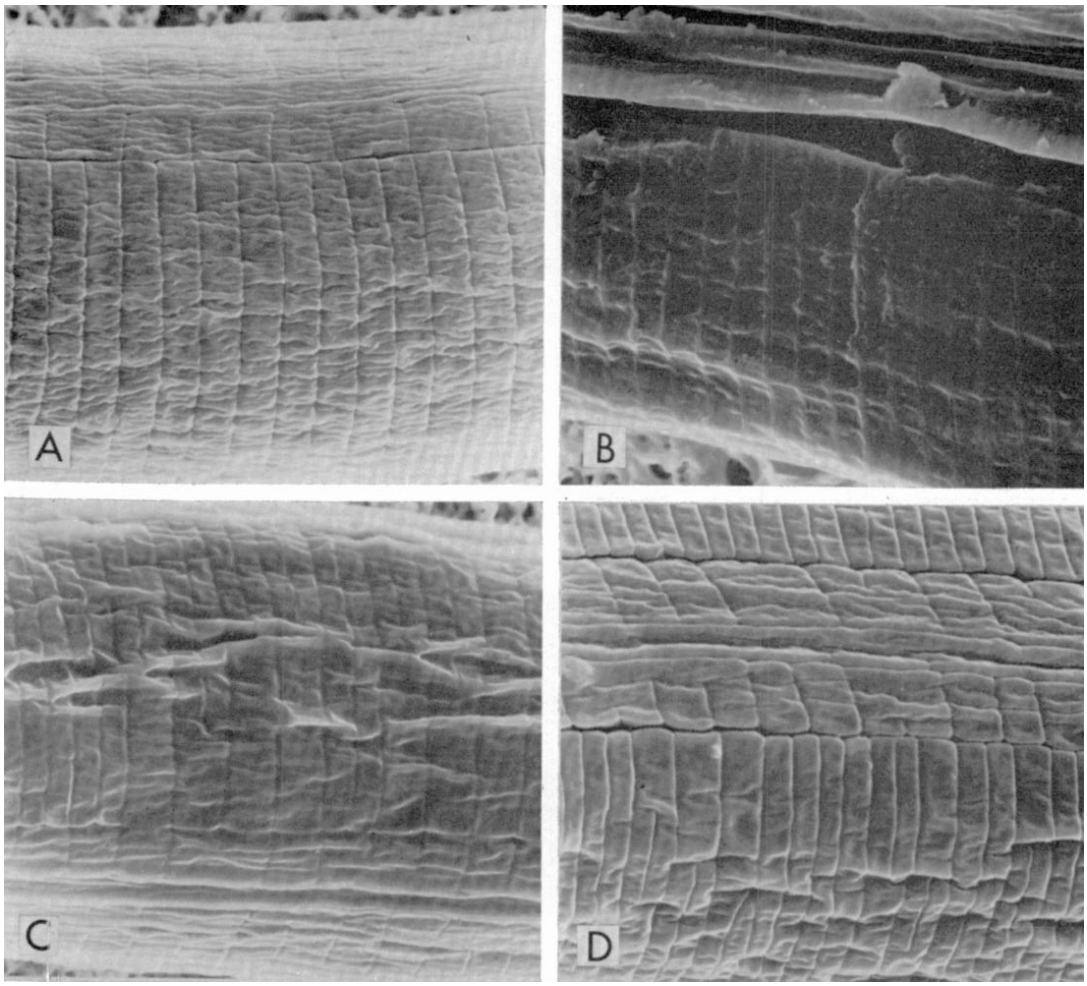

FIG. 1-(A-D). Scanning electron micrographs (5,000 X) of midsection of *Tylenchorhynchus dubius*. *A)* No enzyme treatment; *B)* Chitinase; *C)* Protease; *D)* Lipase.

Rich and Miller (11) previously observed that PCNB applied to soil as a control for fungi had no inhibitory effect on nematodes, and in fact, the nematode popultion was stimulated. Such an increase in nematode populations might occur if the PCNB inhibited proteases that were toxic to *P. penetrans* in soil in the same way that it inactivated papain in this test, although papain is not toxic to *P. penetrans*.

Toxic effects of the enzymes were not always the same in solution and in soil. Factors which may have accounted for these differences include enzyme degradation by other enzymes and adsorption on soil organic matter or particles and soil pH.

The different effects of enzyme treatments on the three species of plant-parasitic nematodes may have related to feeding habit and taxonomic differences. *Pratylenchus penetrans* is a migratory endoparasite, living primarily inside the roots, and is thus exposed to a wide variety of enzymes. *Tylenchorhynchus dubius* is an ectoparasite, exposed only to enzymes in the rhizosphere. It is not surprising that *P. penetrans* might be more resistant to enzymes or have a different resistance than *T. dubius*. Saprophagous nematodes occasionally observed in the soil samples were resistant to the enzyme treatment, possibly because they were constantly in an environment of decaying organic matter and enzymes and only those resistant to these enzymes survived.

Tylenchorhynchus dubius was severely injured, even when it was in contact with enzymes for only 2.5 to 24 h. It is to be expected that *T. dubius* populations might

decrease if they were exposed for several days or weeks in soil with a high content of decaying organic matter which released several different types of enzymes, as well as toxic products of decomposition.

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