A Comparison of Pectinases from *Ditylenchus dipsaci* and *Allium cepa* Callus Tissue

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Abstract: Ground and whole *Ditylenchus dipsaci* maintained on onion callus contain no culturable micro-organisms when tested with five check media. Healthy onion callus does not produce pectolytic enzymes. Pectolytic enzymes are present in infected callus. These enzymes are, however, associated with resident nematodes and not host tissues. These results suggest that *D. dipsaci* is the actual source of the endo-polygalacturonase and endo-pectin-methyltrans-eliminase extracted from them. *Key Words:* Enzyme, Onion.

Whether nematodes produce the pectic enzymes extracted from them is open to question. Studies of nemic pectinases have been made with xenic nematodes (3, 10, 11) and washed nematodes (4). In these studies, however, production of pectinases by contaminating micro-organisms was not excluded. Pectinases have also been extracted from surface-sterilized nematodes (8, 10, 15) and nematodes cultured monoxenically (1, 6, 9, 14). That micro-organisms may have produced the pectinases in these cases is unlikely. Investigation of host tissues as sources of pectinases extracted from nematodes has not been reported, although the obligatory parasitism of plant-parasitic nematodes necessarily suggests the host as a possible alternative source for nemic pectinases.

This paper reports tests of this hypothesis by comparison of pectinases extracted from monoxenically cultured *Ditylenchus dipsaci* and from their food source.

Materials and Methods

Sterility tests: *Ditylenchus dipsaci* from onions reproduced on onion callus in monoxenic culture (14) for 8 weeks at 23 C before being harvested for tests. The nematodes were aseptically extracted from callus tissue with cotton-lined, wire-mesh baskets in 9-cm diameter petri dishes. Infected callus tissue was placed in the baskets and covered with distilled water. After 24 hr at 23 C, 1-ml aliquots of nematodes and extracting water were placed in each of 15 sterile petri dishes. Remaining nematodes were ground 2 min in 50 ml water in a Waring Blendor®. One-ml aliquots of the blender contents were placed in each of 15 petri dishes. Three dishes each of ground and whole nematodes were poured with 14 ml of (i) Nutrient Agar (Difco Laboratories), (ii) NIH Agar (Difco Laboratories), (iii) potato dextrose agar (infusion of 200 g potatoes, 20 g dextrose, 15 g agar, 1000 ml water), (iv) yeast extract agar (10 g sucrose, 2 g yeast extract, 10 g agar, 1000 ml water), and (v) Krusberg's medium (5). Dishes were incubated for 5 days at 23 C. Aseptic technique was employed in all procedures.

Extraction of nematode food source: Test tissues were 10-week-old, uninfected onion callus (*Allium cepa* L., 'Aristocrat'; Asgrow, Inc., Orange, Conn. 06477) grown on Krusberg's medium (5), 10-week-old callus infected for 8 weeks with *D. dipsaci*, infected callus from which nematodes were extracted, and infected callus from which nematodes were extracted and then returned. Culture procedures have been previously described (14). Nematodes were extracted

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from infected callus in Merthiolate solution N.F.® (Eli Lilly, Co., Indianapolis, Ind. 46206) with modified Baermann funnels (14). Before use, extracted tissues were washed thoroughly in sterile distilled water.

In tests involving addition of nematodes to the infected callus from which they had been extracted, infected tissue was placed in Baermann funnels with Merthiolate solution and washed as just described. Extracted nematodes were collected in sterile beakers, sedimented 8 hr at 4 C, and washed four times with sterile distilled water by centrifugation. These nematodes were returned to the callus just before grinding.

Tissue for each test was ground 1:1 (w:v) in cold (4 C) distilled water or cold 0.25 N NaCl solution for 2 min with a Waring Blendor cooled to –20 C. The slurry produced by 2 min grinding was filtered with cheese cloth and centrifuged at 30,000 *g* for 20 min at 5 C. The supernatant liquid was dialyzed 18 hr against two changes of 2 liters of cold, distilled water. Extract protein content was determined by the Lowry method (7) with bovine serum albumin as the standard protein.

Tests for pectinmethylesterase were made with NaCl extracts; all other tests with water extracts.

ENZYME ASSAYS: The ability of extracts of uninfected callus, infected callus, and infected callus extracted of nematodes to degrade sodium polypectate (Sunkist Growers, Inc.) and pectin N.F. (Sunkist Growers, Inc., Ontario, Calif. 91764) was tested in #300 Ostwald-Fenske viscosimeters maintained at 30 C. Reaction mixtures consisted of 4 ml 1.6% sodium polypectate (Napp) or 4 ml 1.2% pectin N.F. (pectin), 1 ml active or autoclaved extract (5 min, 1 atm), and 1 ml distilled water. Napp was buffered at *p*H 4.2 through 7.2 with citrate-phosphate buffer (0.1 M citric acid + 0.2 M dibasic sodium phosphate) or *p*H 8.0 and 8.9 with 0.2 M tris(hydroxymethyl)-aminomethane-HCl buffer (tris-HCl). Pectin was buffered at *p*H 4.0 through 7.0 with citrate-phosphate or *p*H 7.9 and 8.5 with tris-HCl. Mixtures containing pectin were tested with and without 0.003 M $CaCl_2$ added. Enzyme activity was expressed as Relative Activity Units (RA units) per mg protein (2), or as percent viscosity loss.

Viscometric activity of extracts from infected callus to which extracted nematodes were returned was tested with reaction mixtures containing Napp at *p*H 4.2 and pectin at *p*H 8.5 made to 0.003 M with $CaCl_2$.

Release of reducing groups from substrates was detected by Nelson's method (12). Reaction mixtures were identical to those described for viscometric tests. Tests were made with 0.5 ml samples of reaction mixtures at 30 C.

Mixtures used in reducing group tests were assayed also for release of unsaturated end-products by the thiobarbituric acid (TBA) method of Neukom (13). Samples of 0.2 ml were assayed for the compounds.

Tests for pectinmethylesterase (PME) in extracts of uninfected callus, infected callus, and infected callus with nematodes removed were made with a modified continuous titration procedure (14). Three ml of active or autoclaved extract were added to 30 ml of 1.0% pectin adjusted to *p*H 4.0 through 9.0 with 1 M NaOH. To test for NaCl activation of PME activity, the amounts of 0.2 N NaOH consumed in titration were measured for 20 min at 23 C before and after the addition of sufficient crystalline NaCl to make the mixture 0.15 N with the salt. Enzyme activity was expressed in μmoles of NaOH used per mg protein per min.

All tests were replicated.

RESULTS

STERILITY TESTS: There was no growth of micro-organisms on any of the pour plates

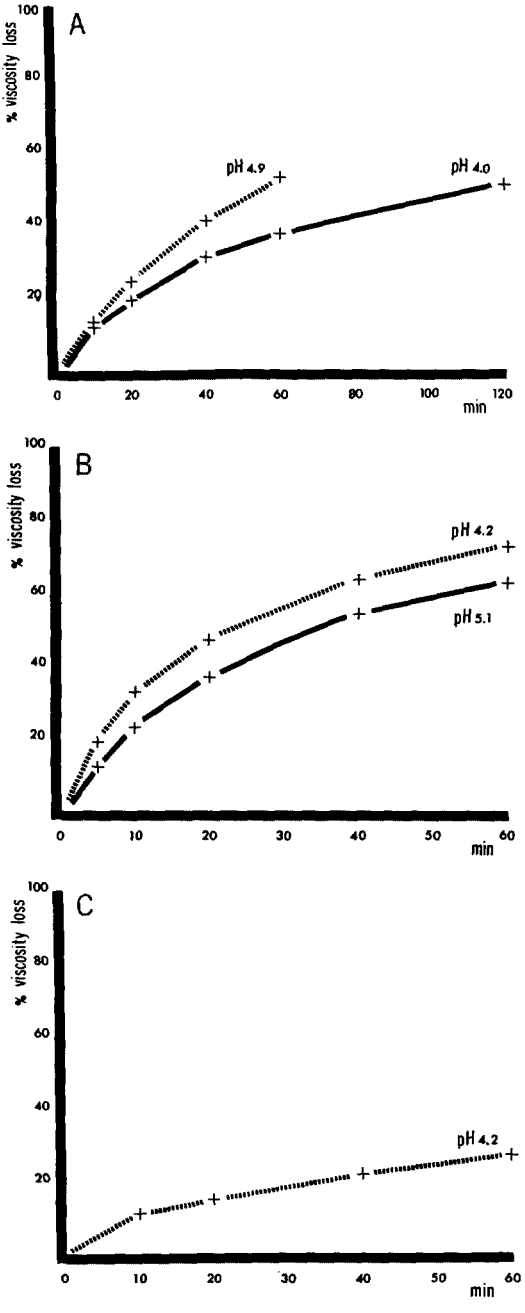

FIG. 1. Viscosity losses of substrates incubated at 30 C expressed as corrected values. **A.** Reaction mixture consisted of 4 ml 1.2% pectin N.F., 1 ml active extract (= 550 μg protein) from infected callus, and 1 ml water; **B.** Reaction mixture consisted of 4 ml 1.6% sodium polypectate, 1 ml active extract (= 550 μg protein) from infected callus, and 1 ml water; **C.** Reaction mixture consisted of 4 ml 1.6% sodium polypectate, 1 ml active extract (= 900 μg protein), and 1 ml water. Extract was made of infected callus extracted of nematodes to which washed nematodes were added.

containing either ground or whole nematodes.

ENZYME ASSAYS: Extracts of infected callus reduced the viscosity of Napp at *p*H 4.2 and 5.1 50% in 23 and 35 min, respectively. At *p*H 4.2 enzyme activity was 79 RA units per mg protein. It was 52 RA units per mg protein at *p*H 5.1 (Fig. 1B). Extract activity on pectin was detectable at *p*H 4.0 and 4.9. At these *p*H values, 50% viscosity losses were recorded in 117 min and 55 min, respectively. Enzyme activity was 16 RA units per mg protein at *p*H 4.0 and 33 RA units per mg protein at p*H* 4.9 (Fig. 1A). $CaCl_2$ caused viscosity increases in substrates during the reaction period.

Extracts of infected callus from which nematodes had been removed were only slightly active in viscometric studies. The initial viscosity of Napp at *p*H 4.2 and 5.1 was reduced about 7% in 60 min. $CaCl_2$ added to pectin caused viscosity increases.

Extracts of infected callus from which nematodes had been removed and reintroduced after washing, actively degraded Napp at *p*H 4.2. The initial viscosity of this substrate was reduced by 27% in 60 min (Fig. 1C).

No viscosity change was detected in reaction mixtures containing extract of uninfected callus.

Release of reducing groups or unsaturated compounds was not detected in reaction mixtures.

All tissues tested contained pectinmethylesterase (Table 1). Extracts of uninfected callus had less PME activity than either infected callus or infected callus with nema-

TABLE 1. Pectinmethylesterase activity in extracts of *healthy* callus, *infected* callus, and infected callus *extracted* of nematodes.

(pH)	μmoles NaOH used/mg protein/min					
	Healthy		Infected		Extracted	
	−NaCl	+NaCl	−NaCl	+NaCl	−NaCl	+NaCl
4	0.005	0.301	0.000	0.312	0.091	0.314
5	0.029	0.512	0.000	0.884	0.107	0.768
6	0.053	0.862	0.000	1.139	0.160	1.338
7	0.177	1.303	0.191	1.348	0.272	1.410
8	0.493	1.023	0.318	1.439	0.346	1.252
9	0.639	0.642	1.273	1.718	0.805	1.249

todes removed. Pectinmethylesterase activity was enhanced by addition of NaCl to the reaction mixture.

DISCUSSION

With a series of media suitable for the growth of a wide spectrum of micro-organisms, contaminating micro-organisms were not detected either in whole nematodes or blended slurries of them. Therefore, that this population of *D. dipsaci* from monoxenic culture was, indeed, associated with only one other organism, the host callus, seems likely.

If the pectinases extracted from the nematodes were produced by the onion callus substrate, they were either performed or induced by the penetration and feeding activities of the nematodes.

Healthy onion callus did not, however, contain detectable pectinases of the type found in the nematodes. Moreover, pectinmethylesterase, the only pectinase detectable in the host, was not extractable from the nematode (14). These data do not support the hypothesis that *D. dipsaci* ingests preformed host pectinases.

The situation is more complex in the alternative case. Since only a few *D. dipsaci* elicit a rather wide-spread reaction in an infected onion plant, 20,000 per culture tube feeding on 1 to 2 grams of callus tissue logically should have stimulated pectinase production throughout the entire callus mass. Thus, in these tests, experimental procedures would have introduced a vast dilution bias favoring the host tissues had they been the actual source of extracted nemic pectinases; that is, host tissues ground 1:1 (w:v) in water should have yielded much more active extracts than nematodes ground 1:25 (v:v) (14). Such was not the case, however, for while infected callus containing nematodes produced active extracts, infected callus extracted of a majority of its nematodes yielded extracts in which pectinase activity was only marginal.

It is possible, of course, that pectinases were removed in the Baermann funnels. Yet, adding a small volume of washed nematodes to such infected callus largely restored activity of pectolytic enzymes. Quite clearly, the pectolytic enzymes were associated with the nematodes and not the infected host callus. And, as before, pectinmethylesterase, though present in even greater quantities in the infected callus, was not found in the nematodes.

Characteristics of the pectolytic enzymes from infected callus strengthens the association of nematodes and the source of enzyme production. This enzyme preferentially attacked pectic acids, viscosity was quickly reduced with apparently a very slow hydrolytic

release of reducing sugars. The enzyme was, then, an endo-polygalacturonase with *p*H optima similar to the endo-polygalacturonase from the nematodes (14). This suggests that the two enzymes could be the same.

The lack in callus extracts of endo-pectinmethyltrans-eliminase, found in this population of *D. dipsaci* (14), was perplexing. Its absence certainly could not support the hypothesis that nematodes ingested pectolytic enzymes from their food source. Neither could it support the opposite, that nematodes produced these enzymes, for it should then have been present in extracts of infected tissues. A possible explanation is the presence of enzyme inhibitors in extracts of host tissue.

While it must be realized that irrefutable evidence for the production of pectinases by plant-parasitic nematodes can only be gained from tests with axenically cultured animals, these data strongly indicate that the test population of *Ditylenchus dipsaci* is capable of forming pectic enzymes and that production of such enzymes by other nematode species is probable.

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