# Pectinases in Aqueous Extracts of *Ditylenchus dipsaci*

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*Abstract:* Aqueous extracts of a population of *Ditylenchus dipsaci* isolated from onion and maintained monoxenically on onion callus contained endo-polygalacturonase (endo-PG) and endo-pectinmethyltranseliminase (endo-PMTE). In viscometric tests *p*H 4.2 and 4.0 were optimal for degradation of sodium polypectate and pectin N.F., respectively, by endo-PG. Endo-PMTE reduced viscosity of pectin N.F. optimally at *p*H 8.5 or above. Activity was dependent on $CaCl_2$. Pectinmethylesterase activity was not detected in water, NaCl, or sucrose extracts of these nematodes. The extracts macerated potato tuber tissue, onion cotyledonary tissue, and strips of onion epidermis from the ventral surface of onion bulb scales at *p*H 4.2, 5.3, and 6.2. Pectin could not be localized with hydroxylamine-ferric chloride reagent in macerated tissues treated for 24 hr with active extract. *Key Words:* Pectinase, Enzyme, *Ditylenchus dipsaci*, Onion.

Production of pectinases by plant-parasitic nematodes has been frequently investigated. Often the criterion for such production has been the presence of pectinases in nematode extracts. Degradation of sodium polypectate by a polygalacturonase present in extracts of *Pratylenchus zeae* was reported (12). Extracts of *Heterodera trifolii* and *Pratylenchus penetrans* were found to degrade pectinic acids (18), and *Meloidogyne arenaria, M. hapla,* and *Radopholus similis* homogenates degraded pectin N.F. (21). A polygalacturonase more specific for sodium polypectate than pectin N.F. was reported in extracts of *Aphelenchus avenae* (2). Tracey (33) reported pectic acid degradation by a polygalacturonase extracted from *Ditylenchus dipsaci.* From *D. dipsaci* infecting alfalfa, Krusberg (15, 16) extracted a polygalacturonase and a pectin-transeliminase, both of which preferentially attacked highly methylated pectins. Muse *et al.* (20) detected endo-polygalacturonase, endo-polymethylgalacturonase, and endo-pectinmethyltranseliminase in extracts of the same nematode. Extracts of a population of *D. dipsaci* from alfalfa (12), and *Ditylenchus triformis* and *Ditylenchus myceliophagus* (9) contained pectinmethylesterase. Pectolytic enzymes were not found in extracts of *Meloidogyne incognita acrita, Aphelenchoides sacchari* and *Panagrellus redivivus* (21), *D. triformis* (2, 12), or *D. myceliophagus* (9).

Secretion or excretion of pectinases by plant-parasitic nematodes has been reported less frequently. Goffart and Heiling (10) claimed a pectinase was secreted into water and oatmeal agar by *Heterodera rostochiensis, Heterodera schactii, Ditylenchus destructor,* and *D. dipsaci.* Myuge (22) reported excretion of protopectinase by *Ditylenchus allii.* Krusberg (15) noted that polygalacturonase was released by *D. dipsaci* incubated in water at 15 C.

The large amount of work involving *D. dipsaci* results from the importance of cell separation relative to nematode feeding and multiplication in host tissues (31) as well as the characteristic tissue maceration symptom in diseases caused by this nematode (3, 7, 8, 22, 23, 25, 28). Maceration of plant tissues has been attributed to dissolution of the middle lamellae of host cell walls (7, 19, 22, 23, 25, 28, 31). Since pectic compounds are important structural components of the middle lamella, pectolytic enzymes have been hypothesized to be agents of dissolution (19, 22, 23). Nevertheless, actual evidence that pectinases are effective in destruction of the middle lamella is scarce and contradictory.

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Myuge (22) claimed a protopectinase excreted by *D. allii* dissolved intercellular protopectinaceous layers and caused tissue maceration. Supportive histological and histochemical evidence was not presented, however. In a later paper he reported retting of flax stems by nematode extracts (23), but his procedures for enzyme preparation did not exclude micro-organisms as possible sources of enzymes.

Krusberg could not macerate alfalfa tissue with extracts of *D. dipsaci* nor could he find histochemical evidence for pectin removal (14). Results of macro-chemical tests also were not indicative of pectin removal. Krusberg (16) concluded that, in this instance, wide-spread degradation of pectic compounds in the middle lamellae of alfalfa plants by nematode pectinases was not a factor in pathogenesis.

Symptoms of onion bloat are in marked contrast to those of the alfalfa diseases, however. In the former, cell separation is commonly observed (22, 23, 26), but cellular death does not immediately follow tissue maceration (23). Since information concerning the pectinases of *D. dipsaci* from onion or the role these enzymes may play in pathogenesis in the onion bloat disease is almost non-existent, further study of the role of nematode pectinases in symptom development is necessary.

This paper reports classification according to the system of Bateman and Millar (6) of pectinases extracted from *D. dipsaci* originally isolated from infected onions. Maceration of the host and non-host tissues by extracts of these nematodes and the removal of pectins from macerated tissues are reported, also.

## MATERIALS AND METHODS

CULTURE PROCEDURES: *D. dipsaci* were derived from a field population infecting onions in Orange County, New York. The nematode had been maintained monoxenically on onion callus. Specimens of the test population are mounted and preserved as entry #2580 in the slide collection housed in the Nematology Laboratory of the Cornell University Plant Pathology Department.

Nematodes for experiments reproduced on onion (*Allium cepa* L. 'Aristocrat'; Asgrow Inc., Orange, Conn.) callus growing on Krusberg's medium (13) dispensed into 25 × 150-mm tubes in 14-ml aliquots. Procedures for callus production and for initiation and maintenance of cultures have been described (30).

EXTRACTION PROCEDURES: Nematodes were removed from 8-week-old cultures with sterile, modified Baermann funnels which could be capped after filling. The contents of five culture tubes and 125 ml of Merthiolate® solution (1 : 1000, Eli Lilly Co.) diluted 1 : 20 with sterile, distilled water were emptied into each funnel. Loaded funnels were sealed in plastic bags and stored under UV light in a transfer chamber for 24 hr at about 23 C. Then, the Merthiolate and the nematodes which had moved to the bottom of the funnel were poured into sterilized, foil-capped 1000-ml beakers. Several 1-ml aliquots from each beaker were examined microscopically for the presence of callus cells. Only breis obtained with nematodes free of micro-organisms and callus cells were used for experiments. Aseptic techniques were followed in handling all materials.

The foil-capped beakers, sealed in plastic bags, were stored 8 hr at 4 C to permit sedimentation of the nematodes. The supernatant liquid was decanted, and the nematodes remaining in the beakers were prepared for enzyme extraction by washing four times in sterile, distilled water by centrifugation. This process yielded 2.5 to 3 ml of fresh nematodes from 100 culture tubes.

The nematodes were ground using 0.45

to 0.50-mm glass beads in a Bronwill Tissue Disintegrator, Model MSK, at 4000 rpm for 30 sec. During operation the apparatus was cooled with gaseous $CO_2$. The brei was washed from the beads with four 10 ml aliquots of cold, sterile distilled water and the combined washings were centrifuged at 30,000 *g* for 20 min at 5 C. The supernatant nematode extract, refrigerated at 4 C, was the test extract.

Protein content of the enzyme extracts was determined with the Lowry method (17) with crystalline bovine serum albumin as the standard protein.

Nematode homogenates were prepared with distilled water for all experiments. In addition, nematodes were extracted with 0.25 M NaCl and 0.25 M sucrose solutions for tests of pectinmethylesterase activity.

ENZYME ASSAYS: Viscosity losses of pectin substrates were measured in #300 Ostwald-Fenske viscosimeters at 30 C. Enzymatic activity was expressed in Relative Activity Units (RA units)/mg protein (5). Reaction mixtures contained 4 ml of 1.8% sodium polypectate (Sunkist Growers, Inc., Ontario, Calif.) or 1.2% pectin N.F. (Sunkist Growers, Inc.), 1 ml of extract (= 825 to 1000 μg protein/ml), and 1 ml water. Controls consisted of reaction mixtures containing autoclaved extract (5 min, 1 atm). Sodium polypectate (Napp) was buffered with citrate-phosphate buffer (0.1 M citric acid + 0.2 M dibasic sodium phosphate) in the *p*H range 3.5 to 7.2 or *p*H 8.0 and 8.9 with 0.2 M tris (hydroxy-methyl)aminomethane-HCl buffer. Pectin N.F. (pectin) was buffered at *p*H 3.0 to 7.0 with citrate-phosphate or *p*H 7.9 and 8.5 with tris-HCl. Mixtures with Napp were enriched to 0.001 and 0.003 M with $CaCl_2$. Mixtures with pectin were enriched to 0.003 M with $CaCl_2$.

Release of reducing groups and unsaturated end-products from reaction mixtures identical to those used in the viscometric tests was measured, respectively, with the methods of Nelson (24) and Neukom (27).

Reaction products from Napp at *p*H 4.3 and pectin at *p*H 4.0 and 8.5 were detected by paper chromatography. Composition of reaction mixtures was the same as described for the viscometry tests. Pectin at *p*H 8.5 was made to 0.003 M with $CaCl_2$. Reaction mixtures were incubated for 24–60 hr at 30 C under toluene. Papers were spotted with 20 μg α-D-galacturonic acid, 20 μg galacturonic acid over-spotted with 40 μl of substrate, 40 μl of substrate incubated with active extract, 40 μl of substrate incubated with autoclaved extracts, 40 μl of substrate,

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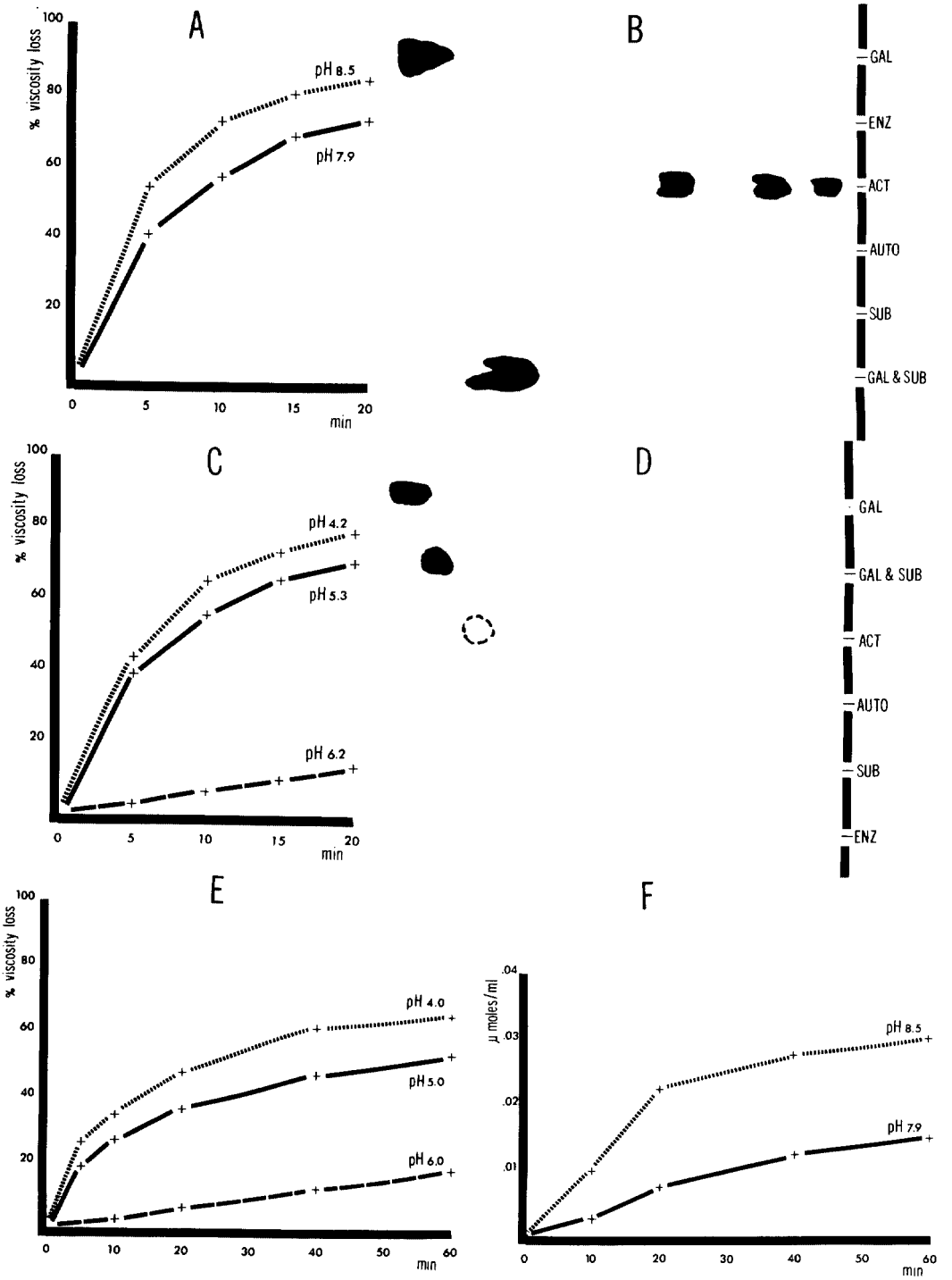
FIG. 1. Enzyme assay of *Ditylenchus dipsaci*. **A.** Viscosity losses of pectin N.F. at *p*H 8.5 and 7.9 at 30 C. Reaction mixtures contained 4 ml 1.2% pectin N.F., 1 ml active extract (= 1200 μg protein), 1 ml water, and 0.003 M $CaCl_2$; **B.** Products released from sodium polypectate at *p*H 4.2 during 24 hr at 30 C. Papers were spotted with 20 μg α-D-galacturonic acid (GAL), 20 μg galacturonic acid + 40 μl substrate (GAL & SUB), 40 μl substrate (SUB), 40 μl substrate incubated with autoclaved nematode extract (AUTO), 40 μl substrate incubated with active extract (ACT), and 40 μl active extract (ENZ); **C.** Viscosity losses of sodium polypectate at *p*H 4.2, 5.3, and 6.2 at 30 C. Reaction mixtures contain 4 ml 1.8% sodium polypectate, 1 ml active extract (= 825 μg protein), and 1 ml water; **D.** Products released from pectin N.F. at *p*H 8.5 during 60 hr at 30 C in the presence of $CaCl_2$. Papers were spotted with 20 μg α-D-galacturonic acid (GAL), 20 μg galacturonic acid + 40 μl substrate (GAL & SUB), 40 μl substrate incubated with autoclaved nematode extract(AUTO), 40 μl substrate incubated with active extract (ACT), 40 μl substrate (SUB), and 40 μl active extract (ENZ); **E.** Viscosity losses of pectin N.F. at *p*H 4.0, 5.0, and 6.0 at 30 C. Reaction mixtures contain 4 ml 1.2% pectin N.F., 1 ml active extract (= 1000 μg protein), and 1 ml water; **F.** Release of unsaturated compounds from pectin N.F. at *p*H 8.5 and 7.9 at 30 C. Reaction mixtures contained 4 ml 1.2% pectin N.F., 1 ml active extract (= 1000 μg protein), 1 ml water, and 0.003 M $CaCl_2$.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and 40 $\mu$l of active extract. Chromatograms were irrigated with 2-propanol: glacial acetic acid solvent (16) for 24 hr at 23–26 C and developed with aniline-diphenylamine reagent (32).

Pectinmethylesterase (PME) activity in extracts was measured with a modified continuous titration procedure (4). Reaction mixtures consisted of 3 ml of extract and 30 ml 1.0% pectin. Acid production at 23 C was measured at *p*H 4 to 9 for 20 min before and after addition of NaCl sufficient to make reaction mixtures 0.15 N with the salt.

Enzymatic macerating of plant tissue was determined using (i) thin disks of potato tissue (*Solanum tuberosum* L. 'Katahdin'); (ii) transverse, free-hand section from cotyledons of 16-day-old onion seedlings (*Allium cepa* L. 'Aristocrat'); and (iii) the epidermal tissue stripped from the ventral surface of onion bulb scales.

Maceration of the above tissues was examined after exposure to the following treatments: (i) 6 ml of distilled water, (ii) 4 ml of buffer plus 2 ml of water, (iii) 4 ml of buffer plus 2 ml of autoclaved extract, and (iv) 4 ml of buffer plus 2 ml of active extract (= 1200 $\mu$g protein). Loss in tissue coherence was determined subjectively by scraping potato disks with a spatula or by teasing onion tissues with dissecting needles. Tissues were examined for maceration after 2, 4, 8, 16, and 24 hr of incubation. Reaction mixtures were buffered at *p*H 4.2, 5.3, 6.2, and 7.2 with citrate-phosphate buffer or at *p*H 8.0 and 8.9 with tris-HCl. At *p*H 8.0 and 8.9, reaction mixtures which had and had not been enriched with $CaCl_2$ to 0.003 M were tested.

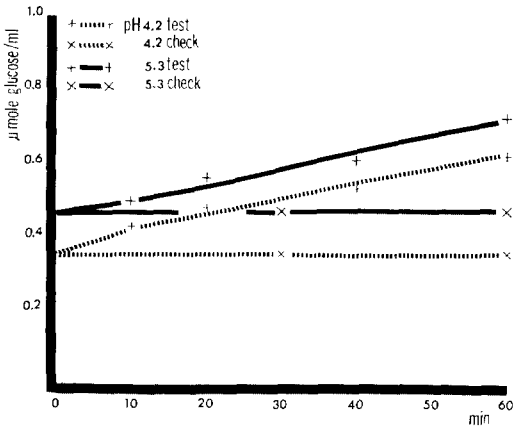


FIG. 2. Release of reducing groups from sodium polypectate at *p*H 4.2 and 5.3 at 30 C measured with Nelson's (24) method. Reaction mixtures contain 4 ml 1.8% sodium polypectate, 1 ml active or autoclaved extract (= 825 $\mu$g protein), and 1 ml water.

PECTIN LOCALIZATION: The above three plant tissues were treated at *p*H 4.3 with a series of reaction mixtures comparable to those described for the maceration assay. Tests were made at 23 C in 6-cm diameter petri dishes. After 0, 8, and 24 hr incubation, two samples of each tissue from each treatment were stained with hydroxylamine-ferric chloride by the method of Reeve (29). One sample was stained immediately after removal from reaction mixtures; the second after methylation with absolute methanol in 0.5 N HCl (29).

Photomicrographs of unmethylated and methylated samples of onion epidermis were made at 0 and 24 hr.

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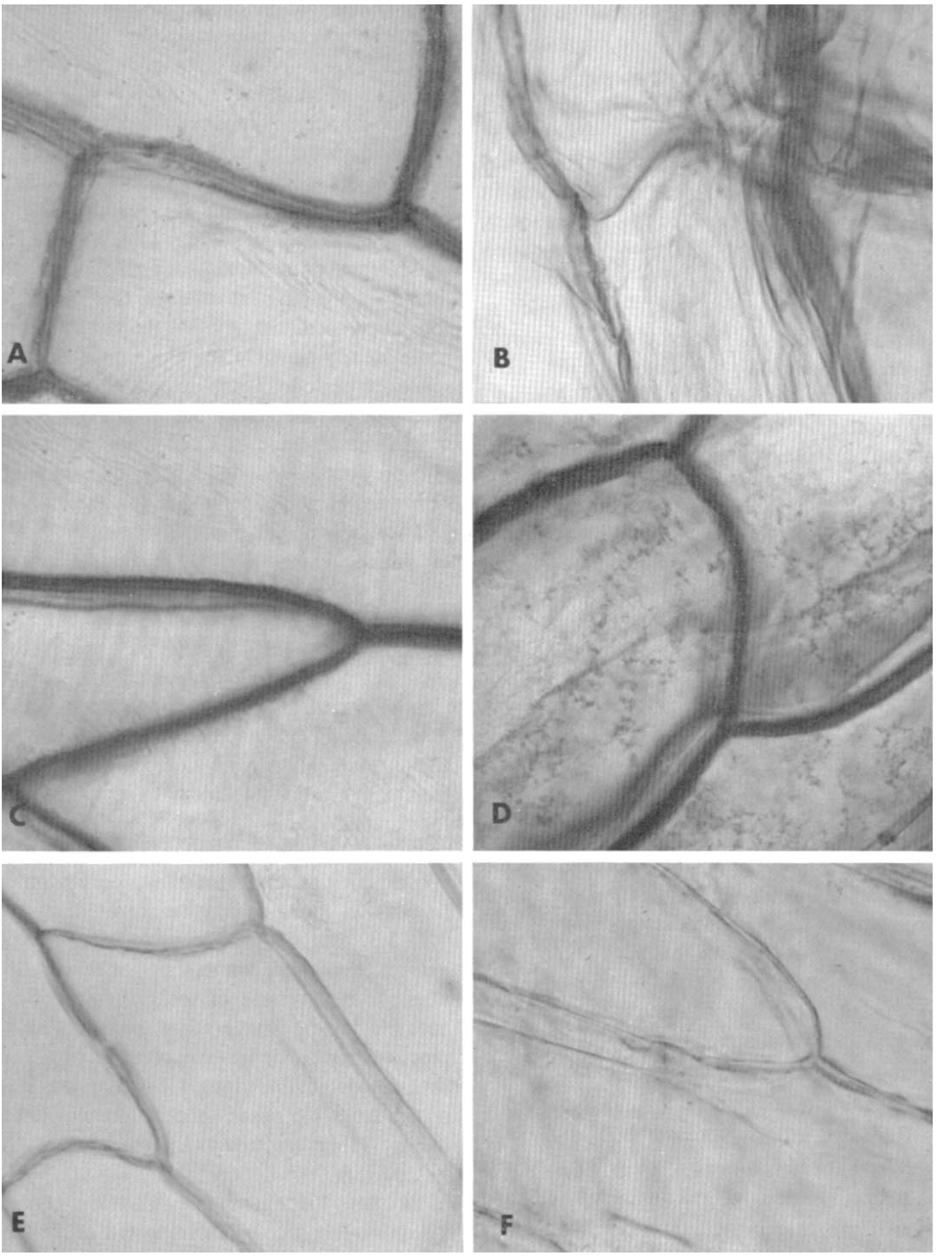
FIG. 3. Localization of pectins in onion bulb scale epidermis with hydroxylamine-ferric chloride reagent. Methylated tissues were treated with absolute methanol in 0.5 HCl before staining. **A.** Fresh unmethylated tissues; **B.** Fresh methylated tissue; **C.** Unmethylated tissue treated 24 hr at 23 C with autoclaved extract; **D.** Tissue treated 24 hr at 23 C with autoclaved extract then methylated; **E.** Unmethylated tissue treated 24 hr at 23 C with active extract; **F.** Tissue treated 24 hr at 23 C with active extract then methylated.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## RESULTS

ENZYME ASSAYS: Detectable losses in viscosity of Napp occurred at *p*H 4.2, 5.3, and 6.2 (Fig. 1C). Enzyme activity at *p*H 4.2 and 5.3 was 187 and 143 RA units/mg protein, respectively. Activity at *p*H 6.2 was much reduced and the point of 50% loss in viscosity was not reached in the reaction period. Addition of $CaCl_2$ to the reaction mixtures resulted in viscosity increases.

In the absence of $CaCl_2$ in the reaction mixtures, losses in viscosity of pectin were recorded at *p*H 4.0, 5.0, and 6.0 (Fig. 1E). Enzyme activity was 42 and 20 RA units/mg protein at *p*H 4.0 and 5.0, respectively. Activity at *p*H 6.0 was too low to permit detection of the point of 50% viscosity loss during the course of the test. $CaCl_2$ stimulated enzyme activity on pectin substrates at high *p*H values (Fig. 1A). At *p*H 8.5 enzyme activity was 178 RA units/mg protein; at *p*H 7.9, 106 RA units/mg protein.

Three reaction products from Napp could be detected after 24 hr incubation at 30 C with active extract at *p*H 4.2 (Fig. 1B). These compounds had $R_{gal}$ values (distance unknown sugar traveled/distance standard galacturonic acid traveled) of 0.416, 0.203, and 0.083. Over-spotted galacturonic acid had an $R_{gal}$ value of 0.674. Degradation products of pectin buffered at *p*H 8.5 in the presence of $CaCl_2$ were detected after 60 hr incubation with active extract (Fig. 1D). The $R_{gal}$ value of this compound was 0.841 and that of standard galacturonic acid over-spotted with substrate, 0.943. The compound did not develop intense color with the developing reagent.

Reducing groups were released from Napp buffered at *p*H 4.2 and 5.3 (Fig. 2). Enzyme activity liberated 33 m$\mu$moles of reducing groups/min/mg protein at *p*H 5.3. At *p*H 4.2, 32 m$\mu$moles of reducing groups/min/mg protein were released. During the reaction period, the release of reducing groups was linear. No release of reducing groups was detected from either substrate buffered at the other *p*H values.

Enzyme activity released unsaturated compounds equivalent to 1.5 and 2.7 m$\mu$moles of the standard/min/mg protein from pectin buffered at *p*H 7.9 and 8.5, respectively, in the presence of $CaCl_2$ (Fig. 1F). At *p*H 8.5 the release of unsaturated compounds was linear for the first 20 min and thereafter the rate decreased. At *p*H 7.9 the reaction was more nearly linearly for the entire 60 min reaction period. Unsaturated end-products were not released from pectin at *p*H 7.9 and 8.5 in the absence of $CaCl_2$, nor were such end-products associated with enzyme activity at lower *p*H values on either substrate.

There was no PME activity in water, NaCl, or sucrose extracts of *D. dipsaci* at any of the *p*H values tested.

Maceration of potato disks was observed after 4 hr at *p*H 4.2 and 5.3 in reaction mixtures containing active extract. Maceration of potato disks in the presence of active extract at *p*H 6.2 was noted after 16 hr. No tissue maceration was noted in any other treatments after 24 hr when the test was terminated.

Similar results were obtained with onion epidermal tissues. However, cotyledonary tissue was more quickly macerated; at *p*H 4.2, 5.3, and 6.2 in the presence of active extract there were some signs of maceration after 2 hr. Because of the difficulty of accurately rating maceration of such small pieces of tissue, definite maceration of tissues was not detectable until 4 hr after the start of the test. No tissue maceration was noted in any other treatments at the end of the test period.

PECTIN LOCALIZATION TESTS: Hydroxylamine-ferric chloride imparted an intense red color to the middle lamellae of cell walls in all three test tissues at zero time. Methyl-

ated tissue stained even more intensely so that transverse cell walls appeared pinkish. Optical sections of radial walls were dark red. The color was most intense in the compound middle lamellae.

Staining reaction of tissues after 8 hr was similar to that of zero time although unmethylated tissues from water, buffer, and active extract treatments stained somewhat less intensely than before. Intense color development was restored by methylation.

At 24 hr tissues from water and buffer treatments stained very lightly. Tissues from the active extract treatment developed almost no color. Methylation restored color development in tissues from water and buffer but not in tissues from active extract. Tissues treated with autoclaved extract stained intensely before and after methylation. These reactions, as observed with fresh bulb scale epidermis and epidermis treated with autoclaved or active extract for 24 hr, are shown in Fig. 3.

## DISCUSSION

Aqueous extracts of this population of *Ditylenchus dipsaci* have two pectolytic enzymes. One of these is active in the acid *p*H range. Optimum *p*H for this enzyme, as judged by results of viscometry tests, is 4.2 on Napp and 4.0 on pectin N.F. Throughout its *p*H range, the enzyme more actively degrades Napp than pectin. The rapid loss of substrate viscosity correlated with slow release of reducing groups from the substrate is indicative of a random, or endotype cleavage of the substrate polymers. Endo-cleavage mechanism is indicated also by the presence of galacturonic acid oligomers in reaction products. The absence of unsaturated end-products suggests hydrolytic cleavage. A known pectinase with these characteristics is endo-polygalacturonase (endo-PG) (6).

The second pectinase in these extracts is active in the alkaline *p*H range. The optimum *p*H for viscometric activity is *p*H 8.5 or above. For activity, it requires a low concentration of calcium and a methoxylated substrate. The rapid decrease of substrate viscosity by this enzyme and the slow release of end-products detected by the TBA and Nelson's tests indicate an endo-cleavage mechanism is operative. Chromatographic detection of end-products with molecular weights higher than $\alpha$-D-galacturonic acid adds some evidence for such cleavage, although the absence of a proper standard sugar lessens the value of evidence from this source. The long incubation period required for production of detectable amounts of end-products correlated with the rapid loss of substrate viscosity again suggests a random cleavage mechanism. Release of unsaturated compounds from the substrate indicates eliminative degradation of pectin. These are characteristics of an endo-pectinmethyltranseliminase (endo-PMTE) (6).

Extracts of *D. dipsaci* from onion macerate host and non-host tissues at acid *p*H values. Endo-PMTE is active only in the alkaline *p*H range. It is not, therefore, involved in tissue maceration. If a nematode pectinase is involved in this maceration, it would necessarily be endo-PG, since maceration occurred at precisely those *p*H values most favorable for that enzyme's activity in viscometric tests.

The hydroxylamine-ferric chloride reagent, considered specific for pectins (11), provides definite evidence of removal of pectic compounds in macerated tissues. The fact that the degree of methylation of pectins has an important influence on color production (29) explains some of the staining reactions observed. Thus, staining of tissues from buffer and water treatments after maceration, but not before, indicates that pectins were not destroyed during the 24-hr test period but rather were demethylated. Pectinmethyles-

terase of host origin could have caused this. However, failure of methylation to restore color in tissues treated with active extracts must indicate that pectins were not present; that is, they had been removed by some agent in the active extract. The only pectolytic enzyme active in nematode extracts in the acid *p*H range is endo-PG.

The relationship of pectin removal to tissue maceration is not clear. On a time basis, pectin removal is not correlated with tissue maceration, since tissues are macerated 20 hr before positive indication of pectin decrease is obtained with the hydroxylamine-ferric chloride reagent. Yet, were tissue maceration effected by an endo-PG, exactly this result would be expected, for the structural integrity of pectic acid could be quickly destroyed by cleavage of only a few internal bonds and remaining high molecular weight pectins would still react with the hydroxylamine-ferric chloride reagent.

The presence of pectins in the cell walls of bloated cotyledonary tissues tends to negate the importance of pectin removal in cell separation in pathogenesis. Again, however, endo-PG activity could destroy the structural properties of lamellar pectins and leave stainable pectins in the cell walls. Moreover, in the disease situation involving intact host tissues the action of massive amounts of enzyme on small pieces of tissues which obtained in the test situation would probably not occur. Nematode enzymes causing tissue maceration *in vivo* would probably act only locally. Blake (8) observed in oats that only cells adjacent to nematodes separate. This would support a hypothesis of localized nematode enzyme action.

Qualitative and quantitative differences exist between enzymes produced by these nematodes and those found by Krusberg (12, 15, 16) in *D. dipsaci* from alfalfa. He detected a polygalacturonase having a marked preference for pectin N.F. at *p*H 6.0. Pectin-methyltrans-eliminase was detected in these extracts, also. Activity of the trans-eliminase was optimal at *p*H 7.0, and it was not, apparently, calcium stimulated. Krusberg (12) reported PME in extracts of this population. Alfalfa tissues were not macerated by extracts of the nematode he studied.

Pectinase activity in extracts of an alfalfa population of *D. dipsaci* studied by Muse *et al.* (20) differ, also. Endo-PG and endo-PMG activity was greatest at *p*H 5 and *p*H 6, respectively. Calcium-stimulated endo-PMTE activity was optimal at *p*H 7.5 to 8.0 in these extracts.

The sources of difference between these studies and the present work could be partially procedural. Ayers *et al.* (1), for example, have shown that relative activity of enzyme preparations or molecular size and initial viscosity of substrates can cause significant shifts in *p*H optima. However, within a test, populations of *D. dipsaci* from alfalfa can differ in the substrate specificity of their pectinases (20). Such evidence suggests innate differences in pectinases between nematodes from onion and those from alfalfa.

It has been suggested that pectinases in nematodes may determine their ability to attack higher plants (16). This generalization assumes particular importance in the specific case of *Ditylenchus dipsaci*, a pathogen frequently associated with host cell separation, for tissue maceration is thought to be directly related to nematode survival. This nematode also possesses an impressive amount of pectinases. For example, in this study, 2 to 3 ml of fresh nematodes ground and diluted in 50 ml of water yielded extremely active extract even when diluted 6-fold. That the enzyme-bearing portion of the nematode must represent only a minute part of the total body volume increases the enormity of dilution. Moreover, populations of *D. dipsaci* which infect different host plants and cause different symptoms in these

plants apparently produce different pectolytic enzymes. All of these factors suggest that in the case of *D. dipsaci,* where closely related populations are segregated by host range, differences in the pectolytic enzyme system could indicate fundamentally different mechanisms of pathogenesis. The importance of pectinases in the pathogenicity of this nematode deserves more study.

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