A Pectin Lyase Inhibitor Protein from Cell Walls of Sugar Beet

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ABSTRACT

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A protein from sugar beet roots inhibited pectin lyase (PNL) that was isolated from *Rhizoctonia solani*. The PNL inhibitor protein (PNLIP) was extracted from healthy sugar beet cell walls and partially purified by ammonium sulfate fractionation, cation exchange, and gel permeation chromatography. Electrophoresis in polyacrylamide with sodium dodecyl sulfate showed a single major band of 57.5 kDa. Isoelectric focusing gave a major band at pI 9.9. Kinetic analysis indicated that the inhibition was uncompetitive (coupling). Pectin lyase inhibitory activity was greater in cell wall extracts from rotted and adjacent tissue than in healthy tissue. The inhibitor concentration was higher in a root rot-resistant germplasm than in a susceptible cultivar and was also higher in root than in hypocotyl or crown tissue. The inhibitor protected root disks from PNL damage in a buffered reaction mixture. The inhibitor was equally effective against PNL from *Phoma betae* but was less effective against PNL from *Aspergillus japonicus*.

Additional keywords: Beta vulgaris, crown and root rot, pectolytic enzyme.

Rhizoctonia solani Kühn (*Thanatephorus cucumeris* (A. B. Frank) Donk) produces several cell-wall degrading enzymes, some of which have been implicated in pathogenesis (6). A pathogenic isolate of this fungus from anastomosis group 2-2 (AG 2-2) produced pectin lyase (PNL) in culture, and PNL was the major pectolytic enzyme detected in sugar beet tissue infected with *R. solani* (10). Extracts of root tissue rotted by *R. solani* contained less pectin lyase activity than extracts from the more susceptible rotted crown or hypocotyl tissue (10). Also, cell walls from crown tissue induced more PNL than did the cell walls from root tissue when walls were used as the source of carbon in broth culture (10). In earlier reports, unexpected low yields of polygalacturonase (PG) from infected tissues of other plants were attributed to proteinaceous inhibitors of host origin (5,17). Fisher et al (18) suggested that plants possess a wide array of pectic enzyme inhibitors and evidence has been accumulating to support this theory (3,5, 8,11,15–17,18). Therefore, the objectives of this research were to see if sugar beet root cell walls contain an inhibitor that affects the production or activity of the PNL from *R. solani* and to purify and characterize the inhibitor.

MATERIALS AND METHODS

Extraction of root tissue. Healthy root tissue of 3- to 4-mo-old, greenhouse-grown sugar beet of the germplasm line FC 712 was the source of extract for purification. The plants were grown in a commercial potting mix (Sunshine Mix 1, Fisons Western Corp., Vancouver, BC, Canada) in 25-cm plastic pots. Root tissue was disintegrated and the cell mass was separated from the liquid phase by centrifugation in a vegetable juicerator (Acme Juicerator Co., Sierra Madre, CA). The cell wall mass was washed in the juicerator with 20 mM potassium phosphate buffer (KPB) (pH 6.8) by continuous flow-through centrifugation until the buffer was clear. The cell wall mass then was collected and washed twice by two 30-sec homogenations in four volumes (w/v) of distilled water in a Waring blender. Water was expressed manually from the homogenate through two layers of cheesecloth. The cell wall preparation was extracted immediately or frozen (−22 C) in lots of 130–150 g. The remaining procedures were done at 4–6 C. The extraction buffer (EB) consisted of: 50 mM KPB, pH 6.8; 1.2 M NaCl; and 0.5% EDTA. Protein was desorbed from the cell walls by mixing approximately 300 g wet weight of cell walls in 600 ml of EB and mixing for 1 h with a motorized paddle at 80 rpm. The homogenate was allowed to sit overnight for protein desorption. The crude extract was filtered through two layers of cheesecloth and the filtrate was centrifuged at 3,000 *g* for 1 h to remove particulates.

Six plants of the root-rot susceptible sugar beet cultivar Ultramono were grown in the greenhouse as above. They were inoculated with barley-grain inoculum of *R. solani*. Barley was soaked 4 h in potato-dextrose broth, drained, and then autoclaved on two successive days. One-fourth of a petri dish culture of *R. solani* AG 2-2 was used to inoculate 250 ml of the sterile barley contained in a 1-L flask. The inoculum was incubated for 2 wk at 25 C and then air-dried and stored at −20 C. The 3-mo-old plants were inoculated by placing barley inoculum around the root 6–9 cm below the soil line. Six plants were inoculated with sterile barley as a control. Six weeks later, the roots were harvested and separated into three tissue categories: rotted portions, the 6–8 mm of tissue adjacent to the rot, and the apparent healthy tissue beyond 8 mm. These three classes of tissue were extracted as described above, and the extracts were assayed for PNL inhibitory activity.

Purification. The crude extract of FC 712 was brought to 25% saturation with ammonium sulfate for 30 min then centrifuged at 3,000 *g* for 30 min. The supernatant was then brought to 85% of saturation with ammonium sulfate and allowed to set overnight at 5 C. The precipitate was collected by centrifugation (3,000 *g*, 30 min) and redissolved in about five times the pellet volume in 20 mM KOH-TES (*N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid) buffer containing 0.1 M NaCl adjusted to pH 7.5. The extract was dialyzed overnight against 12 L of the same buffer. The dialysate was centrifuged (3,000 *g*, 30 min) to remove particulates and then loaded at 25 ml/h on a 1.6 × 30 cm column of CM Sepharose Fast Flow gel (LKB-Pharmacia Inc., Piscataway, NJ) that had been equilibrated with the same buffer. After unadsorbed protein had been washed from the column, as indicated by a UV monitor at 280 nm, the gel was eluted with a linear gradient of 0.1–0.8 M NaCl in the equilibration buffer. Fractions with inhibitory activity were pooled, and the protein was precipitated with ammonium sulfate at 85% of satur-

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ation. The protein was collected by centrifugation, dissolved in 50 mM KPB with 0.4 M NaCl at pH 6.8 and loaded on a 2.6- × 50-cm column of Sephacryl 100 HR that had been equilibrated with the same buffered saline. Eluate fractions with inhibitory activity were pooled, concentrated, and desalted by ultrafiltration with a Centricon unit (Amicon, Beverly, MA) that contained a 10-kDa molecular weight cutoff membrane.

The cell extracts and chromatographic fractions were assayed at room temperature for PNL inhibitory activity by measuring the change in absorbance at 235 nm in a buffered pectin reaction mixture (4). The PNL used in these assays was purified from broth cultures of *R. solani* AG 2-2 (10). The assay reaction mixture contained 10 mM MES, 0.022% citrus pectin (86% methoxy content, Sigma Chemical Co., St. Louis, MO), 1.5 mM $CaCl_2$, 75 mM NaCl, and the pH was adjusted to 6.5. To 2 ml of the buffered pectin in a 1-cm quartz cuvette was added 8–10 units of PNL and 1–50 μl of root extract or chromatographic fractions. The increase in absorption, due to the formation of uronide double bonds, was recorded at 5 min of reaction time. Absorption values of the reaction mixture containing the inhibitor were compared to a control mixture without the inhibitor. The activity rates of PNL and PNL plus wall extract were linear during the first 5 min of the reaction. A unit of inhibition was defined as that amount of protein that decreased PNL activity by 50% in 1 min.

A step to allow binding of the inhibitor to either PNL or the pectin before the assay did not affect inhibitory activity. The same results were obtained whether the inhibitor was mixed with the buffered pectin 5 min before the addition of the PNL or if the PNL and inhibitor were mixed 5 min before the addition of the buffered pectin or if the inhibitor, PNL and buffered pectin were added simultaneously.

Protein concentrations were determined by the colloidal gold method (13) using the reagent supplied by Diversified Biotech, Newton Centre, MA.

Dose response and mode of inhibition. Five levels of inhibitor of 0.5–3 μg, in 0.5-μg increments, were assayed with five units of PNL to determine a dose response curve. Mode of inhibition was measured in a buffered pectin series containing 5 μl of pectin lyase and two levels of inhibitor. The reciprocals of the reaction rates were used against the reciprocals of the pectin concentrations for a Lineweaver-Burke plot to determine the mode of inhibition (28).

Protein nature of the inhibitor. A partially purified inhibitor preparation from FC 712 was exposed to heat and a proteolytic enzyme to determine if the inhibitor was proteinaceous. Two milliliters of a desalted ammonium sulfate fraction of root extract was exposed to boiling water for 10 min and then assayed for activity. For proteolysis, 50 μg (about 0.01 U) of protease XXV from *Streptomyces griseus* (Sigma Chemical Co., St. Louis, MO) was mixed with 69 U of the PNL inhibitor and incubated at 30 C for either 2 or 6 h. In another treatment, phenylmethylsulfonyl fluoride (PMSF) and 50 μg of leupeptin were added to a similar reaction mixture to retard proteolytic activity. The PMSF was added as stock solution in isopropanol to a final concentration of 2 mM. Distilled water containing only the PNL inhibitor was the control. The mixtures then were assayed for PNL inhibitory activity as described previously.

Gel electrophoresis. The purity and molecular mass of the final preparation was estimated by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) using Laemmli's buffer system (23). The gels, 10 × 16 cm and 1 mm thick, were a gradient of 10–20% acrylamide. Electrophoresis was at 15 mA for 300 volt-hours. Prestained molecular weight standards, prepared for silver stain detection, were used as recommended by the supplier (Diversified Biotech, Newton Centre, MA) to monitor protein migration and estimate molecular weights. Glycoprotein determination of resolved bands was done using the modified thymol-sulfuric acid method of Racusen (26) with glycogen as a positive control.

The isoelectric focusing point of the purified inhibitor was determined in a 10 × 16 cm × 0.5 mm vertical slab of 5.5% polyacrylamide. Two ampholytes, one with a pH range of 8.5–10 and the other 9–11, were mixed 1:1 and added to the gel to give a 2% concentration. The gel was polymerized on a polyester film backing (FMC Bioproducts, Rockland, ME) to facilitate handling. Resolution of this basic pectin lyase inhibitor protein (PNLIP) was increased by reversing the normal procedure. The anolyte was 10 mM phosphoric acid and placed in the upper chamber. The catholyte was 20 mM NaOH and placed in the lower chamber. The electrodes were reversed, the positive was connected to the negative power source, and the negative was connected to the positive power source. Electrophoresis was done at constant 20 W with 1,500 V maximum for about 3 h. The catholyte was cooled with a recirculating refrigerated bath set at 5 C. Prestained isoelectric focusing (IEF) standards (Bio-Rad, Richmond, CA) were used to judge the completion of focusing and to estimate the isoelectric focusing point of the inhibitor.

Protein bands after SDS-PAGE were visualized using the silver stain method of Blum et al (7). Protein bands after IEF were visualized with Coomassie Blue R-250.

Effect of pH and NaCl on pectin lyase inhibitor activity. A pH series of 5.5–8.5 in 0.5 unit increments was prepared with 10 mM HEPES buffer containing 0.022% (w/v) pectin, 1.5 mM $CaCl_2$, and 75 mM NaCl. Two milliliters of a pectin solution at a specific pH was placed in a 1-cm quartz cuvette with 5 μl of pectin lyase, and the increase in absorbance was recorded after 5 min at room temperature. Then a purified preparation of the inhibitor was added to the same mixture, and the absorbance was measured again at 5 min. Trials were done in duplicate.

The activity of PNL alone or with a purified preparation of the inhibitor was determined in a buffered pectin solution containing 0–300 mM NaCl in 50 mM increments. The objective was to enhance the assay's sensitivity by using the optimum NaCl concentration.

Content of pectin lyase inhibitor in different tissues. Approximately 10-g tissue samples from crowns, hypocotyls, or roots were homogenized with 50 mM KPB pH 7.5 and 1% ascorbic acid in a Vir Tis tissue homogenizer. The cell mass was washed with 200 ml of the same buffer, collected by filtration and resuspended in for 1 h in 50 ml of the same buffer containing 1 M NaCl to desorb the protein from the cell walls. The cell mass was collected on tared filter paper and the crude filtrate was assayed for PNL inhibitor activity which was expressed as units per milligram of tissue dry weight. Extracts were prepared from 12 greenhouse-grown and eight field-grown plants of root rot-resistant FC 712 and susceptible Ultramono. The data were analyzed by analysis of variance using a completely randomized design with each plant as a replicate. The same analysis also was used on data that were combined from the three tissue types for each cultivar.

Inhibition of PNL damage to root tissue. Cylinders of root tissue 7 mm in diameter were removed with a cork borer from the cultivar Ultramono. One-millimeter-thick disks, averaging 23 mg, were cut from the cylinders with a razor blade. The disks were suspended in two changes of 100 ml of 0.1 M KPB pH 6.8 to remove solutes from the intercellular spaces and cut surfaces. The disks were blotted and one was placed in each well of a 20-well polystyrene tissue culture dish. To each well was added 50 μl of 0.1 M KPB, pH 6.5, together with 100 or 200 U of PNL with or without purified inhibitor. The plate was placed on a Red Rocker (Hoefer Scientific Instruments, San Francisco) at three cycles per minute. The control contained buffer only. Another control to measure maximum cell damage consisted of a disk placed in a microfuge tube with 50 μl of the KPB and exposed to a boiling water bath for 5 min. A syringe needle through the closed cap was used to relieve pressure. Damage to cell and membrane permeability was assessed by measuring the hexose and pentose sugars that diffused into the bathing solution after 1 h of incubation at room temperature. Sugars were measured in 5-μl samples using the orcinol method (21) and quantified from a standard curve using D-galacturonic acid. Treatments were performed in duplicate and the test was repeated.

Association of the inhibitor with resistance. Cell walls of greenhouse-grown roots, 2–3 mo old, were prepared for extraction

TABLE 1. Partial purification of a pectin lyase inhibitor from sugar beet

Step	Vol (ml)	Total activity (units[a])	Total protein (mg)	Specific activity (U/mg)	Purification (×)	Yield (%)
Crude	552	800	70	11	1	100
Cation exchange	42	510	2	255	23	64
Gel permeation	21	476	0.83	573	52	60
Desalted concentrate	0.225	343	0.65	528	48	43

[a] A unit is that amount of inhibitor that reduces PNL activity by 50%.

as described above. Twenty roots each of resistant FC 709 and susceptible F 1010 were prepared. A 10-g sample was taken from each washed cell wall preparation and steeped in 20 ml of EB for 1 h at room temperature. Then 500 μl of supernatant was transferred to a microfuge tube, clarified by centrifugation, and assayed for inhibitory activity. The inhibitory activity was expressed as units per gram of dry weight.

Effectiveness of the inhibitor against PNL from other sources. PNL was purified from liquid cultures of an isolate of *Phoma betae* using the same procedures as reported for *R. solani* (10). This isolate of *P. betae* is pathogenic and causes seedling disease and rot of stored sugar beet roots. A PNL from *Aspergillus japonicus* was purchased from Sigma Co., St. Louis, MO (pectolyase, cat. P-3026). The two sources of PNL were compared with PNL from *R. solani* for their susceptibility to the inhibitor from sugar beet.

RESULTS

Purification and gel electrophoresis. The final yield of the inhibitor was 43% with a purification of 48× (Table 1). An analysis by SDS-PAGE of the final preparation of the inhibitor showed a major band at 57.5 kDa (Fig. 1A). Treatment of electrophoresed gels with thymol-sulfuric acid did not reveal any bands of glycoprotein except for the glycogen that was used as the positive control.

The results with IEF showed a single major band with an estimated isoelectric focusing point of pI 9.9 (Fig. 1B).

The yield of inhibitor from cell wall extracts that were prepared with ascorbic acid and PVPP in the extraction buffer did not differ from the extract that was prepared without those amendments. The inhibitory activity was 11 U/ml for both preparations.

Dose response and mode of inhibition. A nonlinear curve resulted when increasing amounts of inhibitor were plotted against percent inhibition of PNL activity (Fig. 2). When the reciprocals of pectin concentrations and reaction rates were plotted, the result was parallel lines (Fig. 2 insert). This reaction indicated a coupling or uncompetitive type of inhibition (28).

Protein nature of the inhibitor. The ability of a cell wall extract to inhibit pectin lyase was destroyed after the extract was exposed to a boiling water bath for 10 min. Inhibitory ability of a desalted ammonium sulfate fraction of a cell wall extract also was destroyed within 2 h after exposure to protease XXV from *S. griseus*. Inhibition of proteolysis by leupeptin and PMSF resulted in PNL activity that was inhibited 42% after 2 h and 22% after 6 h of incubation. In the control with only the inhibitor, PNL inhibition was 26% after 2 h and 39% after 6 h of incubation.

In addition, pectin lyase inhibitory activity was associated with 280-nm absorbance peaks in the chromatographic elution fractions.

Effect of pH and NaCl on inhibitory activity. As the pH increased from 5.5 to 8.5, the activity of PNL increased ninefold from 97 units to 914 units. In the presence of the inhibitor, PNL activity was reduced 47% at pH 5.5 and 27% at pH 8.5 (Fig. 3). Maximum PNL activity occurred at 200 mM NaCl. When the inhibitor was present in the reaction mixture, the maximum inhibition of PNL was at 100 mM NaCl (Fig. 4).

Content of pectin lyase inhibitor in different tissues. The amount of the inhibitor in the crown, hypocotyl, or root of the resistant FC 712 was higher than in the same tissues of the susceptible

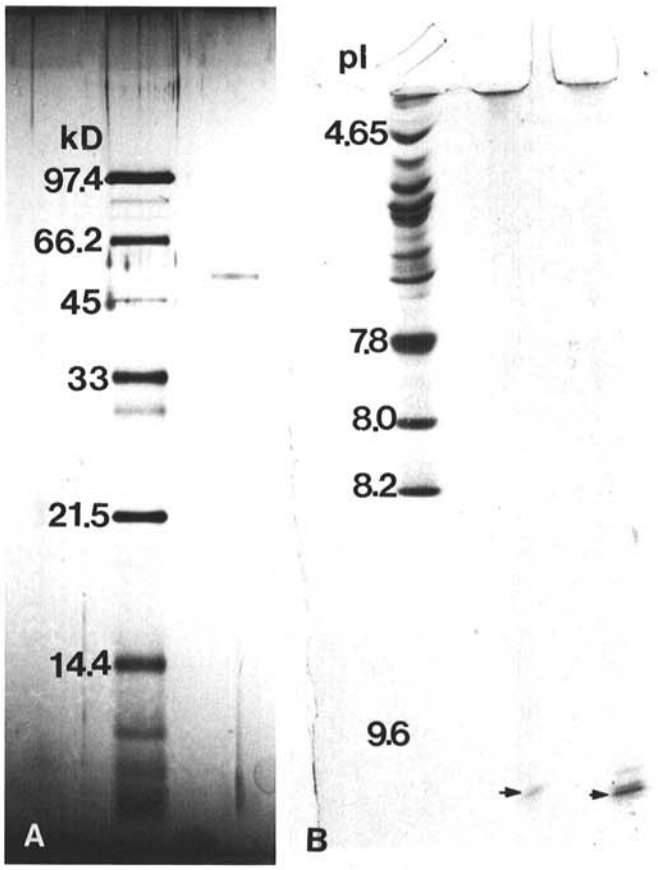

Fig. 1. A, Electrophoresis in a 10–27% gradient polyacrylamide sodium dodecyl sulfate gel of PNLIP purified by ammonium sulfate fractionation, cation exchange, and gel permeation chromatography. Lane 1, molecular mass standards; lane 2, 17 ng of PNLIP. Silver-stained for protein detection. **B,** Isoelectric focused PNLIP at pI 9.9; lane 1, pI standards (cytochrome C at pI 9.6 not visible in photograph); lanes 2 and 3, partially purified PNLIP (arrows) at 8 μg of total protein of adjacent fractions with peak inhibitory activity after gel permeation chromatography.

Ultramono, but the difference was not statistically significant. The inhibitor content was significantly higher in FC 712 than Ultramono when the inhibitor values for all three tissues were combined and analyzed (Table 2).

Crude cell wall extracts from diseased root tissues of Ultramono had PNL inhibitory activities that were two times greater (0.87 U/mg dry weight) than crude cell wall extracts from the adjacent 6–8 mm of tissue (0.44 U/mg dry weight). Extracts from both tissues had greater inhibitory activity relative to the apparent healthy tissue beyond 8 mm from the rot (0.21 U/mg dry weight). The means of the three tissues were significantly different at $P = 0.05$ according to Duncan's multiple range test.

Inhibition of damage to root tissue. When disks of Ultramono root tissue were exposed to PNL, cell damage, as measured by hexose diffusion, decreased as the level of inhibitor was increased. Complete protection (100% inhibition) of 100 units of PNL occurred with 320 units of the inhibitor. When PNL was doubled

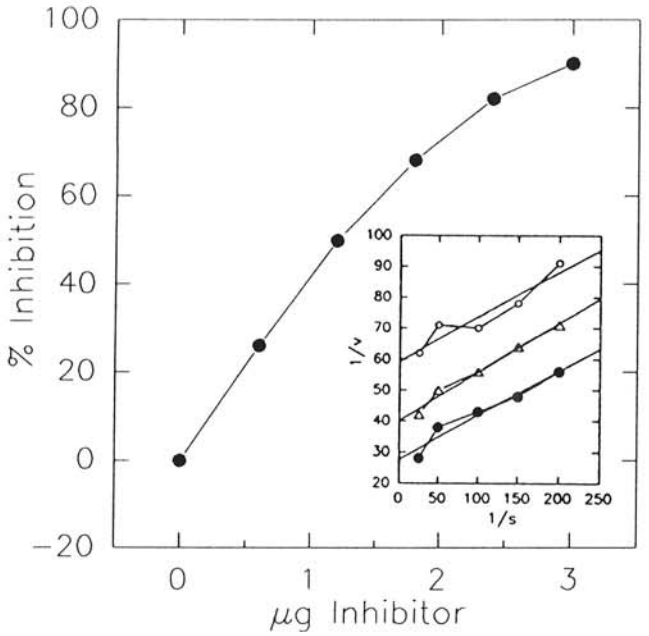

Fig. 2. The percent inhibition of pectin lyase when exposed to increasing levels of pectin lyase inhibitor protein from sugar beet cells. (Insert) Kinetics of the inhibitory activity of a pectin lyase inhibitor protein against pectin lyase. Plot of the reciprocals of rate (V) and substrate (S). Solid circles, PNL alone; triangles, PNL plus 5 μl of PNLIP; open circles, PNL plus 5.5 μl of PNLIP.

to 200 units, 72% inhibition occurred with 320 units of inhibitor (Table 3).

The association of inhibition with root rot resistance. The average PNL inhibitor activity in extracts from the resistant FC 712 was 10 units per microgram dry weight of tissue. The average for the susceptible F 1010 was 2 units per microgram dry weight. The range for F 712 was 3–34 units and F 1010 0–10 units per microgram dry weight. All 20 of the FC 712 samples had inhibitory activity, whereas only half of the F 1010 samples had inhibitory activity. A Student's *t* test analysis showed the two means were significantly different ($P = 0.001$).

Effectiveness of the inhibitor against PNL from other sources. The inhibitor was equally effective against PNL from *R. solani* and *P. betae*, causing a 33% reduction in PNL activity. Inhibition was five times lower against the PNL from *A. japonicus*, causing a 6% reduction in PNL activity.

DISCUSSION

Several accounts of host sources of proteinaceous inhibitors of pectolytic enzymes have been reported. Most of the reports have dealt with PG (1–3,5,6,11,12,14–18,20,24,27,29) and few with PNL (4). My results reveal the presence of a pectin lyase inhibitor protein in cell walls of sugar beet that is active against PNL produced by *R. solani*. The inhibitor was found in higher concentrations in the root than in the crown or hypocotyl, which suggests the inhibitor might have partially accounted for the reported (10) lower PNL yield from infected roots than from infected crowns or hypocotyls.

Inhibitors of PG have been extracted from infected as well as healthy tissues. A proteinaceous inhibitor was complexed with

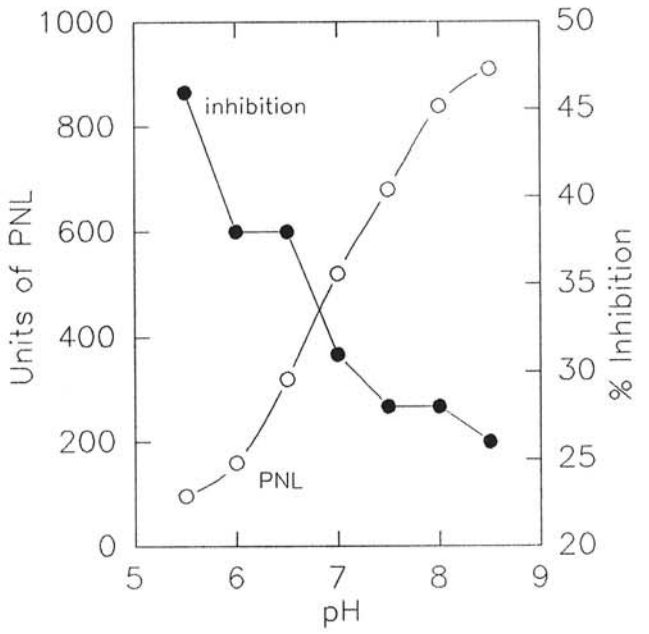

Fig. 3. The effect of pH on the activity of pectin lyase (PNL, open circle) and on the percent inhibition of PNL (solid circle) in the presence of pectin lyase inhibitor protein.

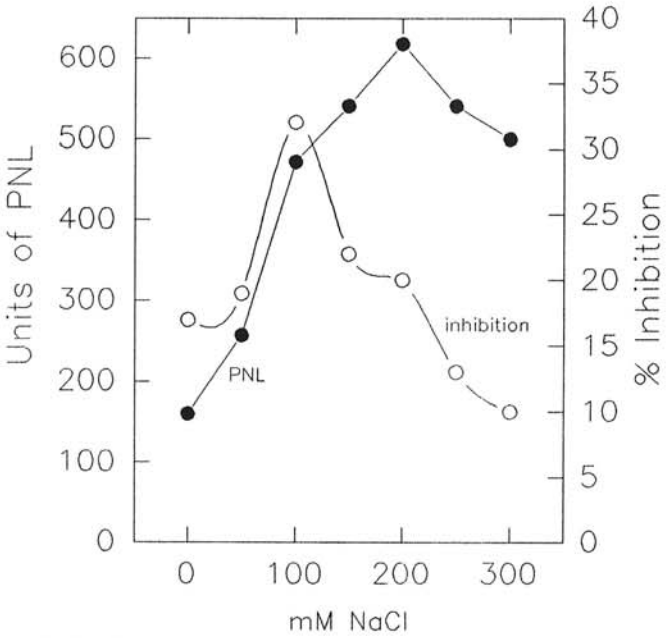

Fig. 4. The effect of sodium chloride on the activity of pectin lyase (PNL, solid circle) and on the percent inhibition by a pectin lyase inhibitor protein (open circle).

TABLE 2. The content of pectin lyase inhibitor protein (PNLIP) in different tissues of *Rhizoctonia* resistant germplasm line FC 712 and the susceptible cultivar Ultramono

Cultivar	Units[a] of PNLIP mg^{-1} tissue dry weight			
	Crown	Hypocotyl	Root	Mean
FC 712 (root-rot-resistant)	72	86	96	84
Ultramono (root-rot-susceptible)	63	62	66	63
F test	NS	NS	NS	*[b]

[a] A unit is that amount of PNLIP that reduces PNL activity by 50%.
[b] Significant, $P = 0.05$.

PG in citrus tissue infected with *Diplodia natalensis* (5). The inhibitor also was detected in healthy citrus tissue but was not quantified (5). A PG inhibitor was detected in peach and plum fruits rotted by *Monilia laxa* or *M. fructigena* but not healthy fruits. The evidence suggested the inhibitor was of host origin but there were no data to distinguish between de novo synthesis and release by pathogen activity (17). An inhibitor of PG was identified in both healthy bean hypocotyl tissue and hypocotyls infected with *Colletotrichum lindemuthianum*. The PNL in the tissue, which was unaffected by the inhibitor, was considered a major factor in the formation of lesions because of the large increase of PNL in infected tissues and its toxicity toward hypocotyl cells (29). In sugar beet roots, the PNLIP content in rotted and adjacent tissue was higher than in healthy tissue. The higher PNLIP levels could have resulted from stimulated synthesis rather than a release from disintegrated tissue because high levels were found in tissue near the rot that had no obvious symptoms. Using monoclonal antibodies against PNLIP revealed higher levels of PNLIP in extracts from diseased than from healthy tissue (W. M. Bugbee, *unpublished data*). This suggests that the inhibitor in the diseased tissue is the same as that in the healthy tissue. Further serological tests are in progress to confirm this finding.

The mode of inhibition, as indicated by the parallel lines in the double reciprocal plot in Figure 3, is interpreted as a coupling mechanism (28). The coupling mode of inhibition, also termed uncompetitive, refers to a three-way binding complex of the inhibitor, substrate and enzyme so that the formation of product is reduced or prevented. A coupling mechanism has been reported for PNL that involves auxin, pectin, and PNL (4). This mode of action would explain why there was no enhanced inhibition when PNL was incubated with the inhibitor or when the inhibitor was incubated with pectin before the assay. The coupling reaction cannot occur until all three components are present in the assay mixture.

The ineffectiveness of ascorbic acid and PVPP would suggest that oxidized phenolic compounds are not involved in this inhibition.

PNLIP was less effective against PNL from *A. japonicus*, a nonpathogen of sugar beet, than against the PNL from the two pathogens, *R. solani* and *P. betae*. The PNL enzymes from the two pathogens are similar in that they have optimum activity at pH 8 to 8.5, whereas the optimum for the PNL of *A. japonicus* is pH 7.

Cultured apple cells were quickly injured and died after treatment with PNL (19,22). Ion leakage was the first sign of cell damage. Leakage of hexoses from living sugar beet root disks exposed to PNL was reduced by PNLIP, showing that the purified inhibitor was effective in protecting living tissue against full damage from PNL (Table 3). Brown and Adikaram (8) showed that a cell wall protein from *Capsicum annuum*, when mixed with pectic enzymes from *Glomerella cingulata*, completely inhibited maceration of the fruit. However, Turner and Hoffman (27) reported that a protein inhibitor of endo-PG was much less effective in preventing cell wall hydrolysis than in preventing the hydrolysis of PG in solution and cautioned against predicting the effect of an inhibitor based on in vitro results.

TABLE 3. Inhibition by pectin lyase inhibitor protein (PNLIP) of hexose leakage from root tissue (cell damage) caused by pectin lyase (PNL)

	Tests			
	1 (100 U PNL)		2 (200 U PNL)	
PNLIP (units)	hexoses (μg)	inhibition[a] (%)	hexoses (μg)	inhibition (%)
0	35	0	75	0
80	44	0	60	20
160	5	86	28	63
320	0	100	21	72
Heat treated[b]	160	...	75	...

[a] The percent inhibition was calculated as the reduction in hexose leakage from tissue treated with purified PNLIP compared to tissue not treated with PNLIP.

[b] A root disk in a glass vial was placed in a boiling water bath for 5 min then removed to the treatment buffer. The treatment without PNL or PNLIP was used to zero the spectrophotometer.

The PNL produced by *R. solani* is most active at an alkaline pH (10,25). The colonization and decay of petioles, crowns, and hypocotyls of sugar beet by *R. solani* is accompanied by an increase in pH from 6.5 (healthy) to 7.5 and occasionally 8, whereas the pH of infected, belowground root tissue decreases to values as low as pH 4, as measured with a surface pH electrode (W. M. Bugbee, *unpublished data*). An increase in PNL activity also was associated with an increase in the pH of extracts from bean hypocotyls infected with *Colletotrichum lindemuthianum* (28). PNLIP was effective at the pH of healthy sugar beet tissue, 6.5–7.0 (Fig. 4). Therefore, the regulatory effect of pH would favor PNLIP initially at the infection site, and then, as the disease progressed, would favor PNL in the alkaline environment of infected crown, hypocotyl, and petiole tissues. During the root rot phase, where rot progresses more slowly, the acid pH of infected root tissue together with a higher PNLIP content would retard PNL activity and contribute toward the slow progress of the AG 2-2 strain of *R. solani*.

Lafitte et al (24) found higher PG inhibitor levels in resistant than in susceptible bean tissue. Brown (9) concluded that pectic enzyme inhibitors regulated tomato rot but not penetration or quiescent infection. Higher PNLIP contents were found in resistant germplasm FC 712 than in the susceptible Ultramono (Table 2). This evidence, together with the protection by PNLIP of root disks against PNL, the increased levels of PNLIP in rotted and adjacent tissues, and the pattern of pH changes in infected sugar beet, suggests that PNLIP has a role in the biochemical resistance of sugar beet to *R. solani*. The significance of this inhibition is being studied, especially with regard to its interaction with other resistance mechanisms.

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