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Short Communication

Role of Pectinesterase in pH-Dependent Interactions between Pea Cell Wall Polymers

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ABSTRACT

Extracts of etiolated pea (*Pisum sativum* L.) shoots converted soluble pectin from the seedlings to a trichloroacetic acid-insoluble form. This activity coincided with pectinesterase peaks separated from the extracts by gel filtration and ion exchange. The conversion of pectin to the trichloroacetic acid-insoluble form and pectinesterase exhibited identical responses to pH, with activity only above pH 6. The formation of trichloroacetic acid-insoluble pectin in pea cell walls and their extracts is due to de-esterification of the pectin by pectinesterase and not to binding between pectin and a protein, as reported in the literature.

Bates and Ray (1) recently reported that the pectin in isolated cell walls of pea seedlings was converted to a TCA-insoluble form when the walls were incubated at neutral pH. The reaction also occurred in water extracts of the cell walls, and was inhibited by Ca^{2+} and by low pH. They concluded that the cell walls contain a protein that binds soluble pectin at pH 7 to form a TCA-insoluble product. However, evidence is presented in the present paper showing that the formation of TCA-insoluble pectin is due to de-esterification of the pectin by pectinesterase.

MATERIALS AND METHODS

Plant Material. Seedlings of *Pisum sativum L.* cv Alaska were grown in moist vermiculite in the dark at 25°C for 7 d.

Extraction of Soluble Polysaccharides. Freshly harvested pea shoots (86 g) were added to boiling water and heated for 10 min. The sample was cooled, homogenized, and reheated for 10 min. The homogenate was then centrifuged at 20,000*g* for 20 min. Three volumes of 95% ethanol were added to the supernatant solution. The precipitate was collected by centrifugation, dissolved in water, and dialyzed against water. Th yield was 78 mg of galacturonan.

Extraction and Chromatography of Pea Pectinesterase. Freshly harvested pea shoots (110 g) were homogenized with 300 ml cold water using a Polytron (Brinkmann Instruments, Inc.). The subsequent steps were conducted at about 3°C. The homogenate was centrifuged at 20,000*g* for 20 min and the supernatant solution was discarded (1). The pellet was suspended in 300 ml of 0.5 M NaCl (pH 6.0), stirred 1 h, and centrifuged. The protein in the supernatant solution was precipitated with $(NH_4)_2SO_4$ at 75% of saturation, collected by centrifugation, dissolved in 15 ml of 0.15 M NaCl, and dialyzed overnight against 0.15 M NaCl.

The dialyzed 0.5 M NaCl extract was concentrated to 10 ml by ultrafiltration using a PM-10 membrane (Amicon Corp.) and applied to a 2.5 × 90-cm column of Sephadex G-100 equilibrated with 0.15 M NaCl. The fractions collected were assayed for pectinesterase, protein, and pectin insolubilization, as described below. Those fractions containing pectinesterase were pooled, concentrated to 10 ml by ultrafiltration, and dialyzed against 0.02 M NaCl. One ml of the solution was applied to a Mono S, HR 5/5 column (a strong cation exchanger) using an HPLC system with a gradient programmer (Pharmacia Fine Chemicals). Buffer A consisted of 0.02 M Mes (pH 6) and buffer B consisted of 0.02 M Mes and 1.0 M NaCl (pH 6). The system was programmed for linear gradient segments of 0 to 2 min (0% B) and 2 to 30 min (0–100% B) at a flow rate of 1 ml/min. The 1-ml fractions were assayed for pectinesterase, protein and pectin insolubilization.

Analyses. Pectinesterase activity was measured at 25°C by titration of the carboxyl groups liberated in 0.2% pectin (Sigma Chemical Co.) containing 0.1 M NaCl in a total volume of 40 ml. The pH was maintained at 8.0 with an automatic titrator which controlled the amount of 0.004 N NaOH added to the reaction mixture. A unit of activity is defined as that amount that catalyzed the release of 1 μmol of carboxyl groups in 10 min under the above conditions.

The conversion of pectin to the insoluble form was measured essentially as described by Bates and Ray (1). The reaction mixture consisted of 1 ml of 10 mM Pipes/acetate buffer (pH 7), 1 ml of 0.1% pectin (pH 7), and 0.1 ml of enzyme solution diluted with 0.15 M NaCl. After 1 h at 25°C, an equal volume of 10% TCA was added to the sample. It was cooled in ice water, centrifuged at 20,000*g* for 20 min, and the supernatant solution was analyzed for uronic acid by the hydroxydiphenyl method (2).

RESULTS

Preliminary studies showed that water extracted a small amount of pectinesterase from pea seedlings. Repeated extraction and washing of the cell walls, especially with low speed centrifugation (1000*g*, 5 min) as described by Bates and Ray (1), gradually solubilized the pectinesterase. However, 0.5 M NaCl was much more effective than water in solubilizing the activity. The procedure developed for isolation of the pectinesterase consists of one extraction with water to remove the cytoplasmic components followed by extraction of the cell walls with 0.5 M NaCl. The salt extract prepared according to this procedure contained 1920 units of pectinesterase.

Chromatography of the salt extract on Sephadex G-100 yielded a relatively broad peak of pectinesterase activity, with a pronounced shoulder on the right side of the peak (Fig. 1). The

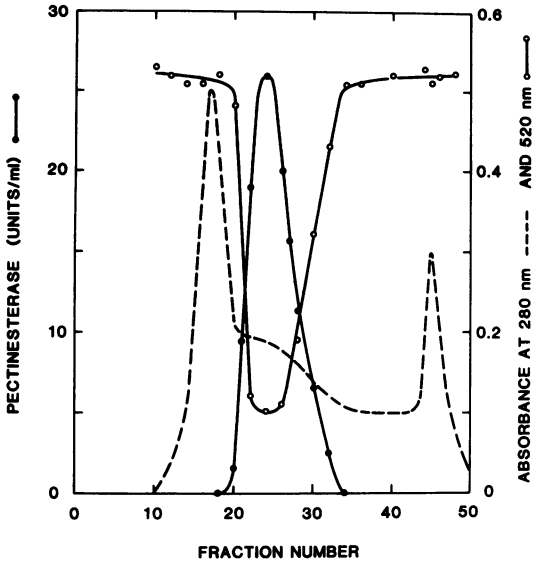

FIG. 1. Elution profile of pectinesterase activity fractionated from a crude extract of pea shoots on a Sephadex G-100 column. The flow rate was 30 ml/h and the fraction volume was 10 ml. Protein in the fractions was monitored by absorption at 280 nm. The solubility of pectin in 5% TCA after treatment with each fraction was measured by the hydroxydiphenyl method (2) (*A* at 520 nm).

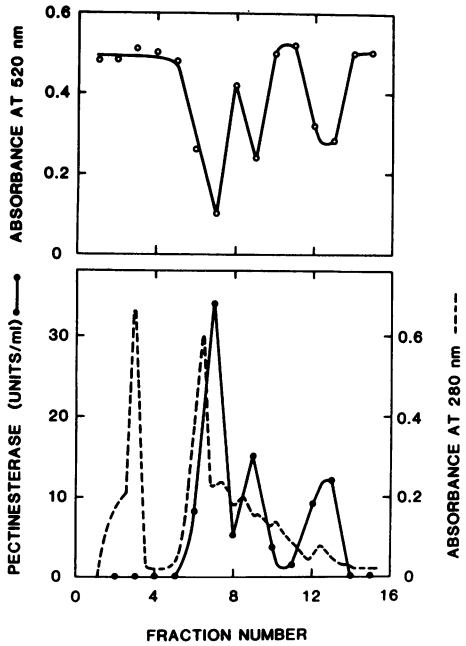

FIG. 2. Resolution of the pectinesterase peak (Fig. 1) into three peaks of activity by chromatography on a Mono S column. The conditions are described in the text, and the fractions were assayed as described for Figure 1.

fractions were also assayed for the conversion of pectin to the TCA-insoluble form. The plot of TCA-soluble pectin remaining in the samples after treatment with the Sephadex G-100 fractions was the inverse of that for pectinesterase activity (Fig. 1). About 20% of the pea pectin remained soluble in 5% TCA even after treatment with an excess of pectinesterase. This soluble fraction may represent low mol wt components of the pectin.

The wide peak of pectinesterase activity obtained on Sephadex G-100 suggested the presence of more than one enzyme. Multiple forms of pectinesterase have been found in other plant sources (7). A complex system of pectinesterase in pea seedlings was

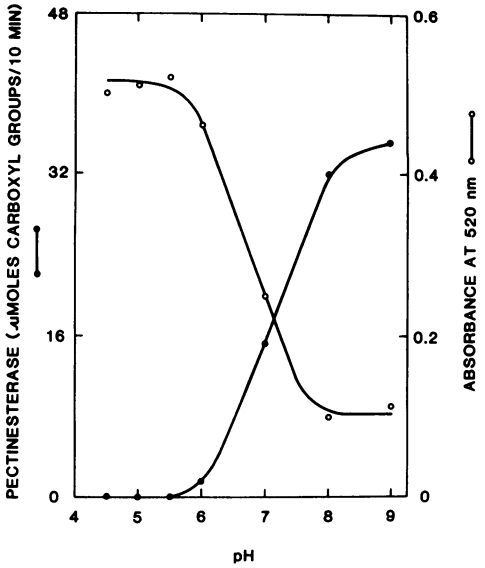

FIG. 3. Effect of pH on pea pectinesterase and on the conversion of pectin to the TCA-insoluble form. The pectinesterase was purified by chromatography on Sephadex G-100.

confirmed when the fractions corresponding to the Sephadex G-100 peak were pooled and chromatographed on the Mono S column. A major peak and two smaller peaks of pectinesterase activity were obtained (Fig. 2). The conversion of pectin to the TCA-insoluble form by the fractions again coincided with the peaks of pectinesterase activity.

Further evidence that pectinesterase is the component in pea cell walls that converts pectin to a TCA-insoluble form was obtained from studies on the effect of pH on the enzyme activity. Reaction mixtures containing 10 mM NaCl were prepared for this study to be comparable in ionic strength with the 10 mM Pipes/acetate buffers used by Bates and Ray (1). At this concentration of NaCl, the pectinesterase was not active below pH 6.0 (Fig. 3). Activity was first detected at pH 6 and it increased sharply as the pH was raised. The pH optimum for the pea enzyme was above 8, which is in agreement with that reported for pectinesterase from other plant sources (5–7). The pea enzyme is similar to other pectinesterase in that the concentration of cations in the reaction mixture had a large effect on the activity and on the pH optimum (6, 7). At pH 7, the activity was increased 3-fold by increasing the NaCl concentration from 10 to 100 mM. Increasing the concentration of NaCl also shifted the activity-pH curves to lower pH values, as reported for alfalfa pectinesterase (6).

Bates and Ray (1) reported that the formation of TCA-insoluble pectin was partially inhibited by 30 mM Ca^{2+}. When assayed with citrus pectin at this level of Ca^{2+}, pea pectinesterase was actually activated. However, both the pectinesterase activity and the conversion of pectin to the TCA-insoluble form were partially inhibited when assayed with the pea pectin. It was observed that 30 mM Ca^{2+} formed a gel with the pea pectin, thus removing it from the action of pectinesterase. This effect on the pea pectin may reflect a lower degree of esterification than for citrus pectin.

Pectin de-esterified nonenzymically by adjusting a 0.1% solution to pH 12 for 15 min was also precipitated by 5% TCA. Furthermore, both enzymically and nonenzymically de-esterified pectins were precipitated by not only 5% TCA but also by acetic, hydrochloric, and other acids. Precipitation of pectate occurred when the pH of its solution was lowered to approximately 4.

DISCUSSION

Bates and Ray (1) observed a conversion of water-soluble polysaccharides, primarily pectin, in cell walls and extracts of

pea seedlings to TCA-insoluble forms during incubation in buffers at pH 7. The responsible factor was heat-labile, TCA-precipitable, and reactive at pH 7 but not at pH 4.5. TCA-insoluble polysaccharide formed at pH 7 did not revert to a soluble form protein that irreversibly binds the pectic polysaccharides at pH 7, rendering them insoluble in TCA.

Another explanation for the formation of TCA-insoluble pectin at pH 7 is that the pectin is de-esterified by pectinesterase to the extent that the acidic polysaccharide becomes insoluble in acids including TCA. The de-esterified pectin can also bind with Ca^{2+} in the insoluble part of the cell wall. The results of the present study are consistent with this interpretation. Pea seedlings contain a high level of pectinesterase, and the enzyme is associated with the cell walls. The conversion of pectin to the TCA-insoluble form by extracts of pea seedlings coincided with the pectinesterase peaks obtained by gel filtration and ion exchange. At the reaction conditions used by Bates and Ray (1) for incubation of the cell walls and extracts, the pea pectinesterase was active at pH 7 but not at pH 4.5. Furthermore, after pectin is de-esterified at pH 7, the change cannot be reversed by lowering the pH to 4.5.

Binding of polysaccharides by proteins may occur in extracts of seedlings (3, 4), but insolubility in TCA (1) is not a valid criterion for such binding because de-esterified pectin is also insoluble in 5% TCA.

Yamaoka and Sato (8) recently reported the presence of a 'pectin-gelling factor' in soybean hypocotyls. This factor, a protein which was released from the cell walls by salt solutions, produced gels from apple pectins in the presence of Ca^{2+}. Its properties were consistent with that of pectinesterase, and Yamaoka *et al.* (9) subsequently confirmed that the 'pectin-gelling factor' was actually pectinesterase. Nevertheless, Yamaoka and Chiba (10) suggested that gradual de-esterification of pectin by pectinesterase resulting in changes in gelling properties may be important in interactions between cell wall polymers associated with plant growth.

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