Purification and Certain Properties of Pectin trans-Eliminase from Aspergillus fonsecaeus*

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In 1960 Albersheim, Neucom, and Deuel (1) reported on an enzyme present in a commercial pectic enzyme preparation (Pectinol R-10, Rohm and Haas Company, Philadelphia) that degraded the \( \alpha,1 \rightarrow 4 \)-glycosidic bonds in pectin by a trans elimination of the proton on the 5th carbon atom of an anhydro methyl galacturonate unit with the oxygen of the adjacent glycosidic bond. Cleavage of the bonds in pectin resulted in the formation of esters of galacturonides of undetermined size with unsaturated bonds between carbon atoms 1 and 5 at the nonreducing ends of the fragments formed. These double bonds strongly absorbed light at 235 nm. The enzymatic process was thought to occur by a reaction mechanism similar to that of the degradation of pectin in neutral (2) or in alkaline (3) solutions. In a more recent publication (4), the purification of pectin trans-eliminase of Pectinol R-10 was described. By the use of diethylaminoethyl cellulose chromatography, Sephadex gel filtration, and isoelectric precipitation, a 22-fold purification was obtained, although the removal of polygalacturonase and pectinesterase from the purified enzyme was not demonstrated.

Similar enzymes, which are specific for polygalacturonic acid, have been reported in cultures of Bacillus polymyxa (5, 6) and in species of Erwinia (7, 8). The present work describes the production of pectin trans-eliminase by Aspergillus fonsecaeus, a fungus which formed this enzyme extracellularly in significant amounts. The purification and some of the properties of pectin trans-eliminase are reported, and it will be shown that this enzyme differs in several respects from bacterial polygalacturonic acid trans-eliminase.

**EXPERIMENTAL PROCEDURE**

**Preparation of Adsorbents**—Calcium pectate gel was prepared from pectin N.F. (purchased from Sunkist Growers, Inc., Corona, California) by the method of Newbold and Joslyn (9). The washed gel was suspended in distilled water to give a concentration of 6 mg of matter, dry weight, per ml.

Calcium phosphate gel was prepared according to the procedure of Kunitz (10). The final concentration was 25 mg per ml. Cellulose \( N,N \)-diethylaminoethyl ether (DEAE-cellulose) was obtained from Eastman Kodak Company (No. 7392) and was converted to the chloride form with 1 M Tris-HCl buffer at pH 7.5. One gram (original basis) of a aqueous suspension was placed in a 1.1-cm chromatography tube; the final height of the adsorbent was 7 cm. Enzyme solutions were dialyzed against 0.005 M Tris-HCl buffer at pH 7.5 before they were placed on the column. Elution was done with an apparatus similar to that described by Palmer (11), and a convexly increasing gradient was used in buffer strength. A constant pressure of 80 cm of water was maintained on the column.

**Preparation of Substrates**—A polygalacturonic acid (No. 491) of high purity was purchased from the Exchange Lemon Products Company, Corona, California. It was used without further treatment.

Pectin N.F. was purchased from Sunkist Growers, Inc.

A soluble pectin, which gave a water-clear solution (suitable for spectrophotometric measurements), was prepared by treating pectin N.F. with yeast endopolygalacturonase as described by Luh and Phaff (12). Yeast endopolygalacturonase, which contains no pectinesterase, effects a maximal hydrolysis of about 1% of the glycosidic linkages and renders insoluble a small amount of amorphous gummy material. Purified yeast endopolygalacturonase (13), 1250 units, was added to 2.5 liters of 2% pectin N.F. in 0.05 M sodium acetate buffer at pH 4.5. After 3 days at 30°, the solution was heated to 80° for 15 minutes to destroy the enzyme and then filtered through diatomaceous earth. To the clear filtrate, sufficient 95% ethanol was added to bring the alcohol concentration to 70%. The precipitate was removed by filtration on a Buchner funnel, dissolved in water, and reprecipitated with ethanol. This suspension was filtered, and the precipitate was washed with 95% ethanol, absolute ethanol, and ethyl ether. The white flakes were dried in air and then in a vacuum over calcium chloride. Thirty-four grams of this material were obtained from 50 g of pectin N.F.

Polymethyl polygalacturonate methyl glycoside was prepared from polygalacturonic acid by the method of Morell, Baur, and Link (14, 15). The ash content of this product was 0.48% (moisture-free basis), and its degree of esterification was 95.5%. According to Jansen, MacDonnell, and Ward (16), its degree of polymerization is about 30.

**Assay Methods**—One unit of polygalacturonase is the amount of enzyme that releases 1 μmole of reducing groups per minute at pH 4.5 and 30° from polygalacturonic acid. The substrate concentration was 0.5% (weight per volume) in 0.1 M sodium acetate buffer. The increase in aldehyde groups was measured at 240 mp. The enzymatic reaction was thought to occur by a reaction mechanism similar to that of the degradation of pectin in neutral (2) or in alkaline (3) solutions. In a more recent publication (4), the purification of pectin trans-eliminase of Pectinol R-10 was described. By the use of diethylaminoethyl cellulose chromatography, Sephadex gel filtration, and isoelectric precipitation, a 22-fold purification was obtained, although the removal of polygalacturonase and pectinesterase from the purified enzyme was not demonstrated.

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between polygalacturonase and pectin trans-eliminase activities, containing 2% pectin N.F. and 0.67% Bacto-yeast nitrogen base.

The enzymes were purified by the method of Lowery et al. (18). Crystallized bovine serum albumin was used to prepare standard curves. For determination of total protein nitrogen, the modification of the Kjehldahl method as described by Johnson (19) was used with bovine serum albumin as a standard. In a few instances the protein content was also measured by the method of Warburg and Christian (20), which compares the ultraviolet absorptions at 260 and 280 μm.

**RESULTS**

Selection of Suitable Fungus—Nine fungi that were known to possess pectolytic activity were screened for their ability to produce extracellular pectin trans-eliminase. To produce spores, the fungi were first grown for 3 or 4 days on potato agar with 0.5% d-glucose. A spore suspension of the various fungi was sprayed aseptically on the surface of a liquid pectin medium. After 5 days of surface growth, the culture fluids were filtered and tested for activity. The results are shown in Table I. Growth was approximately equal in all cases. On the basis of its superior trans-eliminase production, Aspergillus fumigatus was chosen for further work.

Course of Enzyme Excretion by A. fumigatus—Next, excretion of trans-eliminase was followed as a function of time. The course of excretion of polygalacturonase and the protein content of the culture fluid were measured also, so that changes in specific activity could be calculated. When the mold had formed a cover face deck, samples were withdrawn periodically, after the flasks had been gently swirled. The results are shown in Fig. 1. Pectinesterase was demonstrated qualitatively to be present at the end of the growth period. The initial pH of 3.4 of the culture medium always decreased to 2.8 by 2.9 after about 5 days (depending somewhat on the rate at which the mold covered the surface of the liquid) and then rose to 3.6 to 3.9 after 7 or 8 days. Since the specific activity of the trans-eliminase decreased beyond 6 to 7 days of growth, and at the same time the ratio of trans-eliminase to polygalacturonase became lower, further work was done with 7-day-old cultures.

**Purification of Pectin trans-Eliminase**

Since pectin trans-eliminase...
and polygalacturonase would interfere with a study of the properties of trans-eliminase, purification of the culture fluid was directed at removal of these two activities. Before various adsorbents were used, the culture fluid was dialyzed against 40 volumes of 0.005 M acetic acid at 3°C for 20 hours. At this low temperature, the dialysis tubes survived the action of cellulase, which destroyed the cellophane at room temperature. During dialysis the pH of the enzyme solution (3.6 to 3.8) decreased to 3.5.

Patel and Phaff (21) showed that calcium pectate gel completely adsorbed tomato polygalacturonase. The culture liquid was therefore treated with this gel in an attempt to remove polygalacturonase. Calcium pectate gel, 5% by volume, was added to the dialyzed culture fluid at room temperature, and the mixture was stirred for 20 minutes. The gel was then removed by filtration under gravity. It was found that 90% of the polygalacturonase was removed by the gel, whereas about 90% of the original trans-eliminase activity was present in the clear filtrate (see Table II).

Next, calcium phosphate gel, 5% by volume, was added to the enzyme solution adjusted to pH 7.5 with 0.1 M NaOH. The mixture was stirred for 1 hour at room temperature and centrifuged. The supernatant liquid contained no activity and was discarded. The packed gel was dispersed in a small volume of 1 M sodium acetate buffer at pH 5.2. The supernatant was then added to the dialyzed culture fluid at room temperature, and centrifuged after 24 hours. Only about 60% of the trans-eliminase activity was recovered in the supernatant liquid, but the treatment did result in a substantial increase in specific activity (Table II). The concentrated enzyme solution was then dialyzed at room temperature against 0.05 M sodium acetate buffer at pH 5.2. Judging by the strength of the dialysis tubing after dialysis of the concentrated enzyme solution, the cellulase of the original culture fluid had been eliminated at this stage.

Preliminary experiments showed that the trans-eliminase activity of the dialyzed solution was adsorbed completely by a suspension of DEAE-cellulose and that the enzyme could be eluted quantitatively by 1 M sodium acetate buffer at pH 5.2. Next, a column of DEAE-cellulose was used. The concentrated enzyme was introduced at the top, and after the column was washed with water, the enzyme was subjected to gradient elution with a constant volume of 250 ml in the mixing chamber. The two liquids were water and 1 M sodium acetate buffer at pH 5.2.

Fractions of 7.5 ml were collected at room temperature with a constant volume fraction collector. The individual fractions were analyzed for protein concentration by measuring ultraviolet light absorption at 280 mÅ. The trans-eliminase activity was determined in every fifth fraction to locate the enzyme. Under these conditions, the enzyme was eluted from the column between 0.6 M and 0.8 M acetate buffer (Table II).

The tubes containing significant activity (about 90% of the peak area) were combined and dialyzed against 0.1 M sodium acetate buffer, pH 5.2. This material constituted the purified enzyme, which was essentially free of pectinesterase and polygalacturonase. In the most highly purified enzyme solutions (eluates from DEAE-cellulose), the enzyme concentration was suitable for the optical assay, but the protein values were too low to give reliable results with any of the methods employed. The combined active fractions (90% of the peak area) were therefore dialyzed and readsorbed on DEAE-cellulose and were then eluted in a small volume of 1 M acetate buffer. With this solution three methods of protein analysis were used, all of which indicated a comparable final degree of purity. Because the methods are affected differently by the various nitrogenous components present, it is not surprising that the extent of purification varied from 40-fold (for the Lowry method) to 140-fold for the Kjeldahl procedure.

Substrate Specificity—Of particular interest was whether or not trans-eliminase could attack polygalacturonic acid. A concentrated, purified enzyme preparation was incubated with polygalacturonic acid in the presence of 0.001 M CaCl₂ at pH 5.2 and at pH 8.0. Calcium chloride and the reaction mixture at high pH were included since these conditions were shown to be optimal for the bacterial trans-eliminases referred to in the introduction. No changes in absorbance or in reducing value could be demonstrated. The specificity of our purified enzyme for pectin confirms a similar conclusion by Albersheim et al. (1) for a crude fungal enzyme preparation.

Effect of Various Ions on Activity of trans-Eliminase—Since the activity of bacterial polygalacturonic acid trans-eliminase was shown to be dependent on calcium ions (5, 6), the effect of calcium, magnesium, and sodium ions on the activity of pectin trans-eliminase was tested at pH 5.2. All of the cations were added as their chlorides to pectin M, and the results are presented in Fig. 2.
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During the purification of the enzyme, assays were done in 0.1 M sodium acetate at pH 5.2. Under these conditions the sodium ion concentration was 0.08 M. The maximal activity, with 0.13 M calcium chloride, was about 2.5 times as high as the activity measured under our standard assay conditions.

Next, the effect of different anions was studied. Fig. 3 shows the activity of trans-eliminase in relation to different anions of sodium with increasing sodium ion concentration. At high concentrations, phosphate and acetate ions exhibited significantly lower activities than did sulfate or chloride. However, at the sodium acetate concentration used for standard enzyme assays (0.1 M), acetate exhibited no significant inhibition as compared to the sulfate or chloride ions.

Optimum pH—The optimum pH of a crude preparation of trans-eliminase was reported to be between pH 5.1 and 5.3 (1). This value was confirmed with our purified enzyme in sodium acetate buffers (Fig. 4). As it is not uncommon for the optimum pH of an enzyme to shift in the presence of different ionic species, the effect of pH was determined in reaction mixtures with Tris acetate buffer (0.04 M with respect to Tris) and also in this buffer with 0.5 M NaCl or 0.12 M CaCl₂ added. The results are shown in Fig. 5. The fact that the high peak near pH 8.5 in the presence of CaCl₂ was not found in the presence of sodium chloride demonstrated that the increase in optical density was not due to alkaline degradation of pectin (2, 3). The possibility was considered that the peak at pH 8.5 might be due to a second enzyme in the reaction mixture, which could have a specific calcium requirement. However, when the ratios of the activities of trans-eliminase at pH 5.2 and at pH 8.5 were determined at all of the stages of the purification procedure, they were found to be constant. Thus, it is probable that the activities at pH 5.2 and 6.5 were due to the same enzyme.

Chemical Evidence for Formation of Reaction Products with 4,5-Unsaturated Terminal Gatacturonide Units—Thus far the evidence for the formation of these double bonded products was based on the production of compounds showing a progressive increase in absorbance and a maximum at 235 mμ.

As pointed out by Linker, Meyer, and Hoffman (23), only unsaturation in the C-4,5 position of terminal uronic acid units would lead to formation of oxalic acid after treatment with ozone. A solution of 1 g of Link pectin in 130 ml of sodium acetate buffer at pH 5.2 was prepared, and 20 ml of concentrated purified trans-eliminase were added. After 24 hours no further in-
crease in absorbance at 235 μM took place. This reaction mixture was subjected to ozonization at 0°C for 60 minutes with pure oxygen entering the ozonator. Ozonization at room temperature, as recommended by Linker et al. (23), was shown to result in excessive oxidation of the oxalic acid formed. The reaction mixture was evaporated under a vacuum to 20 ml, acidified with 2 M H₂SO₄ to pH 1.2, and heated on a boiling water bath for 30 minutes to hydrolyze remaining methoxyl groups. After cooling, the residue was extracted with diethyl ether, and after drying with Na₂SO₄, it was evaporated to dryness. The residue was taken up in water, neutralized to pH 5.5, and precipitated with calcium acetate. The precipitate was treated with sulfuric acid and re-extracted with ether as above. After evaporation, needle-shaped crystals resulted. An infrared spectrum, with a Perkin-Elmer No. 221 spectrophotometer, was determined with authentic oxalic acid as control. The two spectra were identical, and the crystals obtained appeared to be nearly pure.

**DISCUSSION**

The limited survey of several members of the Aspergillus niger group showed that all species produced pectin trans-eliminase, but in different amounts. The activity figures given in Table I are relative to the presence of pectinesterase in the crude culture fluids. Pectinesterase competes with trans-eliminase for pectin by de-esterifying the substrate, and thus by making it unavailable to trans-eliminase. However, the complete absence of an increase in optical density at 235 μM may be taken as evidence that trans-eliminase is not produced by certain fungi, such as Penicillium chrysogenum. Similarly, the culture fluid of Saccharomyces fragilis (13), which contains yeast endopolygalacturonase, was devoid of trans-eliminase. Albersheim et al. (1) also reported considerable variation in the trans-eliminase activity of a few commercial pectin enzyme preparations and its absence in a purified preparation of fungal polygalacturonase. Even in A. fonsecaeus, trans-eliminase is not the main pectic enzyme produced. Before purification, there was approximately 5 to 6 times as much polygalacturonase as trans-eliminase (Table II). This ratio depends greatly on the time at which the culture liquid is separated from the mold deck (Fig. 1).

The activation of trans-eliminase by cations and the changes in the pH dependence caused by cations must be considered together (cf. Figs. 2 and 4). Cations, and especially divalent cations, have a strong affinity for the free carboxyl groups of partially esterified pectin. Pectin N.P. (or pectin M), with only 68% of its carboxyl groups esterified by methanol, tends to enter into some form of ionic bonding with cations. This has the effect of progressively reducing the negative charge on the substrate at pH values above 5.2. It is noteworthy that even in the presence of 0.5 M NaCl, the activity of trans-eliminase at pH 8.5 was still 30% of that in the presence of 0.12 M CaCl₂, whereas in 0.04 M Tris-acetate buffer no activity was found at pH 8.5 (cf. Fig. 4). The pH-activity curve given by Albersheim et al. (1) also shows considerable activity at pH 5.0 in 0.2 M McIlvaine's buffer. This buffer is a mixture of 0.2 M disodium phosphate and 0.1 M citric acid.

The reported stimulation of trans-eliminase activity by McIlvaine's buffer at pH 5.2 (4), as compared to sodium acetate buffer, was confirmed by us, as was the inhibitory action of calcium in the presence of McIlvaine's buffer.

It is possible that the enzyme polymethyl galacturonase obtained by Seegmiller and Jansen (24) from the commercial enzyme preparation Hydrolase (Nupro Chemical Company, Harrison, New Jersey) constitutes the same enzyme as the one described in this paper. This postulation was supported by our demonstration that a recently purchased sample of Hydrolase contained trans-eliminase activity.

Similar cases of enzymatic cleavage of polymers resulting in the formation of reaction products that strongly absorb ultraviolet light at 230 to 235 μM have been reported (20, 29). In the case of bacterial hyaluronidase (25), the reaction mechanism must be a cis elimination, as the linkage involves glucuronic acid derivatives. By the use of H₂SO₄, Ludwig, Vennesland, and Dorfman (27) showed that bacterial hyaluronidase catalyzed cleavage of its substrate by a direct elimination reaction rather than by an initial hydrolysis followed by elimination of water. In the case of alginic acid breakdown by a bacterial enzyme (26), the mechanism is less clear, as alginic acid contains L-guluronic acid as well as D-mannuronic acid. With the former uronide, trans elimination is possible, but with the latter uronide only cis elimination can occur. It was shown by Fischer and Dorfél (28) that in brown algae the ratio of L-guluronic acid to D-mannuronic acid may be as high as 2:1. Thus, the most unequivocal case of trans elimination so far known involves either pectin or polygalacturonic acid. Enzymes that catalyze elimination cleavage of polysaccharides could be grouped together with enzymes that catalyze elimination of water from simpler substrates, e.g. fumarase and aconitase.

**SUMMARY**

After an initial survey of several fungi, a study was made of the production and purification of pectin trans-eliminase from the culture fluid of Aspergillus fonsecaeus. The fungus was grown as a surface culture on a liquid, pectin-containing, mineral medium. The enzyme was purified by a three-step procedure to the point at which other pectic enzymes and cellulases were no longer detectable. It has been shown that this enzyme is specific for pectin and that it differs from a polygalacturonic acid trans-eliminase produced by certain bacteria. Pectin trans-eliminase was stimulated, in decreasing order of effectiveness, by calcium, magnesium, and sodium, but maximal activity was observed at different cationic concentrations. In high concentration, acetate and phosphate ions were somewhat inhibitory as compared to chloride or sulfate ions. In the presence of 0.1 M sodium acetate, the enzyme showed an optimum pH at 5.2. In the presence of an optimal concentration of CaCl₂ (0.13 M), the enzyme exhibited an activity plateau centered at pH 5.2 and a high peak of activity at pH 5.5. It appears that the activity stimulated by calcium ions at pH 5.5 is due to the same enzyme as the one that has maximal activity at pH 5.2 in the presence of sodium ions.

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