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THE RELATIONSHIP AND PROPERTIES OF PECTIC GLYCOSIDASES PRODUCED BY HOST AND PATHOGEN DURING ANTHRACNOSE DISEASE OF AVOCADO

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Abstract—Polygalacturonase activity was substantially induced in avocado fruit after infection with *Colletotrichum gloeosporioides*. The enzyme was extracted from healthy and infected tissues of inoculated fruits and purified about 13-fold by DEAE-cellulose column. Both enzyme preparations gave a single fraction after elution from the column, though thermal inactivation and disc electrophoretic studies indicated two components in the enzyme fraction of the infected tissue as compared to a single component in the fraction of the healthy one. The two components were quantitatively separated by CM-cellulose chromatography and characterized as fungal and avocado polygalacturonases, respectively. The fungal polygalacturonase constitutes approximately 16 per cent of the total activity in the infected tissue and was not detected in the healthy mesocarp. The fungal enzyme attacked pectic acid by random mechanism of hydrolysis and showed higher activity with non-methylated pectic substrates. Digalacturonic acid was not attacked by the fungal polygalacturonase. The pH optima of the last enzyme was 5·5 and 4·5 for pectic and trigalacturonic acids, respectively. The avocado polygalacturonase showed lower stability to temperature inactivation and higher K_m value as compared to the fungal enzyme. The results demonstrated that softening of the mesocarp during anthracnose development is primarily due to induction of the avocado polygalacturonase.

INTRODUCTION

POLYGALACTURONASES (pectic glycosidase, poly α-1,4-galacturonide glycano hydrolase E.C. 3.2.1.15) are involved in tissue maceration during pathological manifestations by biotic agents.[1,2] Although these enzymes are widely distributed amongst microorganisms, only in a few instances have they been detected in healthy plant tissues.[2-5] The presence of polygalacturonase in avocado fruits has been demonstrated by McCready and McComb[6] and further investigated by McCready *et al.*[7] and Reymond and Phaff.[8] Polygalacturonase could not be detected in unripe Fuerte avocados, though ripe avocados showed high polygalacturonase activity directly related to the extensive decrease in the molecular size of pectin.[6]

Anthracnose is a common disease of avocado fruit caused by *Colletotrichum gloeosporioides* Penz. This disease is characterized by sunken black spots on the fruits and as the fruit ripens the fungus penetrates deeper into the mesocarp until most of the fruit rots. This

[1] R. K. S. WOOD, *Ann. Rev. Plant Physiol.* **11**, 299 (1960).
[2] D. F. BATEMAN and R. L. MILLAR, *Ann. Rev. Phytopathol.* **4**, 119 (1966).
[3] A. L. DEMAIN and H. J. PHAFF, *Walerstein Lab. Commun.* **20**, 119 (1957).
[4] T. A. BELL, *Botan. Gaz.* **113**, 216 (1951).
[5] D. S. PATEL and H. J. PHAFF, *Food Res.* **25**, 47 (1960).
[6] R. M. MCCREADY and E. A. MCCOMB, *Food Res.* **19**, 530 (1954).
[7] R. M. MCCREADY, E. A. MCCOMB and E F. JANSEN, *Food Res.* **20**, 186 (1955).
[8] D. REYMOND and H. J. PHAFF, *J. Food Sci.* **30**, 266 (1965).

symptom is apparently accompanied by a separation of host cells due to destruction of the middle lamella structure.[9]

The information on changes in pectic enzymes of host origin during pathogenesis is very scanty. Pectin methylesterases are present in healthy tissues of many plant species[3] but in larger amounts in diseased tissues.[2] Since pathogens are also capable of releasing pectin methylesterases,[2] the origin of these enzymes in diseased tissue has not as yet been definitely established. The limited distribution of polygalacturonases in higher plants apparently contributed to the general concept that polygalacturonases produced in infected tissues originate from the pathogen, rather than from the host, as a result of host–pathogen interaction. Induction of polygalacturonase elaborated by the host during fungal infection of kaki fruits[10] is the only example of such interaction of which the present authors are aware.

The work reported here describes the purification, properties and relationship of host and fungal polygalacturonases during anthracnose development in the avocado.

RESULTS

Relation of Polygalacturonase to the Degree of Infection and Ripeness

Tissue plugs were removed at different time intervals for polygalacturonase determination, both, from invaded and uninvaded (i.e. opposite to the infection site) areas, of inoculated fruits and from sound fruits. The plugs were taken with an 11 mm cork borer and extracted as described in the Experimental. Results given in Fig. 1 show that polygalacturonase activity was detected in the infection site within 24 hr of inoculation. Enzymatic activity in the uninvaded areas of inoculated fruit was developed after 2 – 3 days and only detectable in control fruits after 7 days.

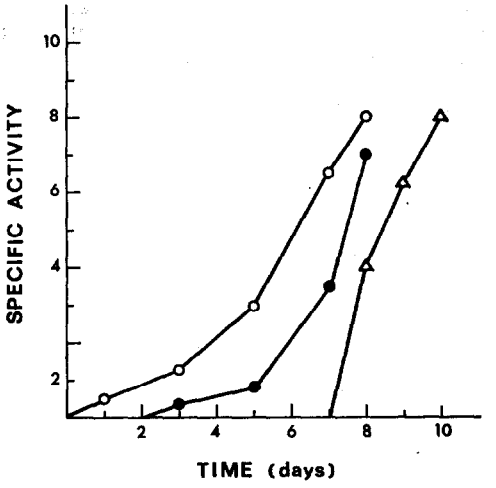

FIG. 1. POLYGALACTURONASE ACTIVITY DURING THE RIPENING STAGES OF INOCULATED AND HEALTHY AVOCADO FRUITS.

Each figure represents an average result taken from three different fruits. Polygalacturonase is expressed as specific activity (enzyme units/mg protein). ○—○, Invaded tissue; ●—● uninvaded tissue from inoculated fruit, △—△ healthy fruit.

[9] N. BINYAMINI, Ph.D. Thesis, The Hebrew University, Jerusalem, p. 93 (1968).

[10] T. TANI, in *The Dynamic Role of Molecular Constituents in Plant Parasite Interaction* (edited by C. J. MIROCHA and I. URITANI), p. 40, Bruce Publishing, St. Paul, Minnesota (1967).

Fractionation and Purification of Host and Fungal Polygalacturonases

Approximately 100 units (0·9 units/mg protein) of crude enzyme preparation obtained from infected tissue was placed on a DEAE column and washed with 100 ml water. Gradient elution was carried out with 150 ml 0·05 M phosphate buffer, pH 8, in the mixing chamber and 150 ml 0·4 M acetate buffer, pH 5·2, in the other chamber. Fractions of 8 ml were collected. Enzyme activity was determined by incubation of 0·5 ml eluent from each fraction with the reaction mixture for 30 min. Under these conditions 90 per cent of the original polygalacturonase activity was recovered in a single peak (Fig. 2). The fractions which contained enzyme activity were pooled, dialyzed against distilled water for 16 hr and then assayed for enzyme activity. The specific activity of the final preparation was 12·5 units/mg protein, a purification of about 13·5 times.

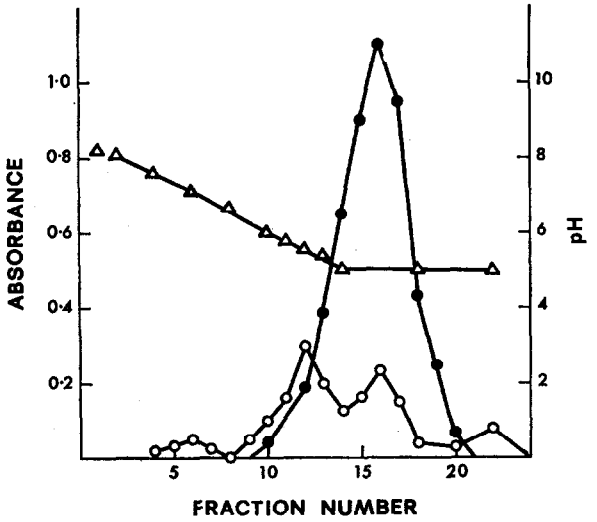

FIG. 2. CHROMATOGRAPHY OF POLYGALACTURONASE FROM INFECTED AVOCADO TISSUE ON DEAE-CELLULOSE.

●—●, Enzyme activity expressed as absorbance at 575 nm after reaction with DNS; ○—○, proteins estimated by extinction at 280 nm; △—△ pH.

A previous report[8] indicated that avocado polygalacturonase was not adsorbed by carboxymethylcellulose (CMC); in the hope that CMC might retain fungal polygalacturonase if present in infected tissue, the dialyzed enzyme, after elution from DEAE, was passed through such a column. Of 258 enzyme units introduced at the top, only 42 units were retained by the ion exchange after additional washing with 100 ml 0·05 M acetate buffer, pH 4·5. Elution of the enzyme from the CMC column was achieved when the concentration of NaCl approached 0·2 M (Fig. 3). All the activity of the eluted enzyme was recovered in a single peak. A crude polygalacturonase preparation, extracted from healthy parts of inoculated fruits, did not lose any activity on the CMC column.

A large amount of fungal polygalacturonase was obtained by growing the fungus on autoclaved avocado slices for 4 days at 25°. The enzyme was extracted from the slices, dialyzed and adsorbed onto CMC. The elution pattern of the enzyme activity was identical to that described in Fig. 3 and resulted in a purification of approximately 12-fold.

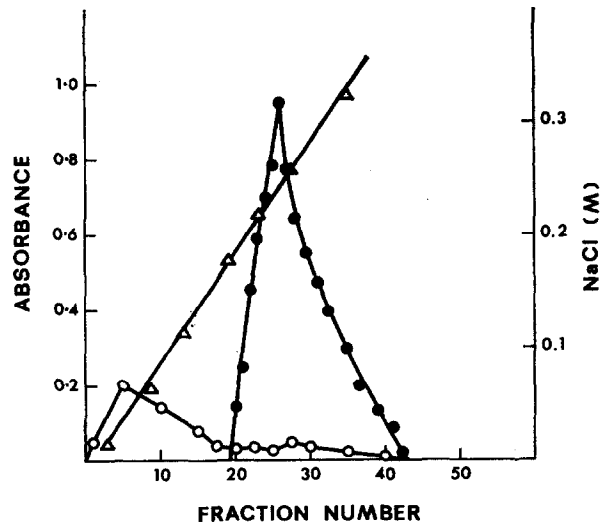

FIG. 3. ISOLATION OF FUNGAL POLYGALACTURONASE FROM THE TWO ENZYME COMPONENTS OF INFECTED TISSUE BY CM-CELLULOSE COLUMN.

Details are given in the text. Enzyme activity and proteins are expressed as in Fig. 2. ●—●, Polygalacturonase activity; ○—○, proteins; △—△ NaCl concentration.

Further evidence of the presence of two components in the infected tissue was obtained by disc electrophoresis. The enzyme in the peak detected after the elution from the DEAE column (Fig. 2) was dialyzed and concentrated by passing through Diaflo ultrafiltration membrane type UM-10 (Amicon, Holland). A purified fungal enzyme preparation from autoclaved avocado slices was also similarly treated. 200 μl of each enzyme preparation were placed in a different acrylamide tube for a running period of 30 min. The results (Fig. 4) indicate the presence of two components in the preparation of infected tissue: the first component corresponding to the fungal enzyme.

Action Pattern and Properties of Fungal Polygalacturonase

Since information on avocado polygalacturonase became available,[6–8] further experiments were aimed at the characterization of the fungal enzyme. Fungal polygalacturonase after CMC column purification was used in the following experiments. The optimum pH with acetate buffer for polygalacturonic and trigalacturonic acids was 5·5 and 4·5 respectively. Reducing group liberation and viscosity changes were followed by pectic acid reaction mixtures (Fig. 5). Immediately after the onset of the reaction, liberation of reducing groups proceeded linearly and, after reaching approximately 35 per cent hydrolysis, rates proceeded to decrease to about 65 per cent hydrolysis within 2 hr. Monogalacturonic acid could be detected only during the second phase of hydrolysis and accounted for splitting of 9 per cent of the galacturonide bonds. The appearance of low-molecular weight oligogalacturonides during pectic acid hydrolysis was followed by paper chromatography.[11] Tri- and digalacturonic acids were detected on chromatograms only at the end of the linear phase of hydrolysis and were later followed by the appearance of monogalacturonic acid. A rapid decrease in relative viscosity was also observed. (Fig. 5).

[11] I. BARASH, *Phytopathology* **58**, 1364 (1968).

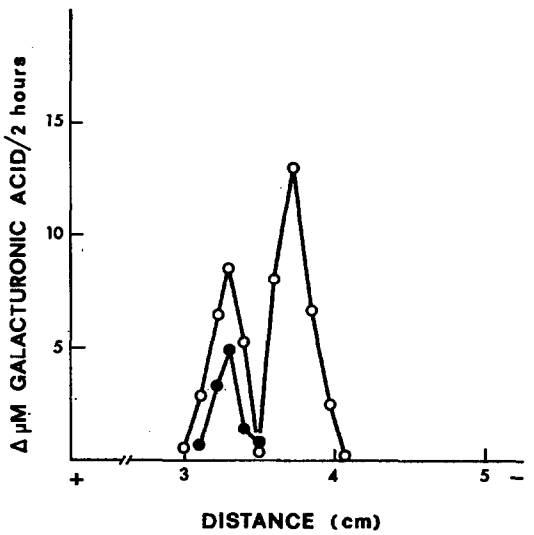

FIG. 4. ELECTROPHORETIC SEPARATION PATTERN OF PARTIALLY PURIFIED POLYGALACTURONASES.
○—○, Enzyme preparation of infected tissue after DEAE cellulose chromatography (Fig. 2);
●—●, fungal polygalacturonase after CMC chromatography.

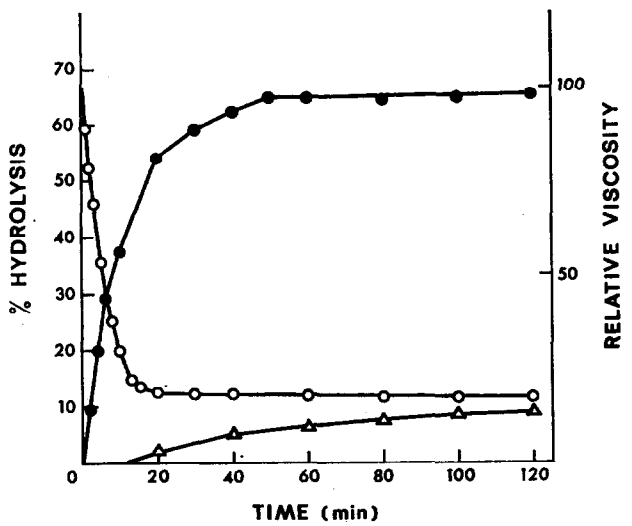

FIG. 5. HYDROLYSIS OF PECTIC ACID BY THE FUNGAL ENZYME AS A FUNCTION OF TIME.
●—●,Total hydrolysis, measured by liberation of reducing groups with DNS; △—△, formation of free galacturonic acid as measured with naphthoresorcinol reagent; ○—○, reduction in relative viscosity. Relative viscosity is defined as the ratio of substrate viscosity to viscosity of the buffer solution in which it is dissolved.

The effect of chain length and esterification percentage of substrates on the activity of fungal polygalacturonase was investigated. The relative rate of hydrolysis of pectic acid (DP = 112), polygalacturonic acid (DP = 12·7), esterified pectic acid, esterified polygalacturonic acid and trigalacturonic acid was 25, 20, 10, 6 and 1, respectively. Digalacturonic acid was not hydrolyzed after incubation at various pH for 4 hr.

Fungal enzyme preparations obtained from infected avocado tissue or from autoclaved avocado slices were found to have identical properties. Attempts to detect polygalacturonate trans-eliminase and pectinesterase in the purified fungal preparation, produced negative results.

Effect of Temperature Inactivation and Substrate Concentration on Avocado and Fungal Polygalacturonases

The stability of the partially purified polygalacturonases from invaded and uninvaded tissues (after DEAE column) or fungal enzyme (after CMC column) at various temperatures is given in Fig. 6. 1 ml portions of enzyme were heated at indicated temperatures for 10 min

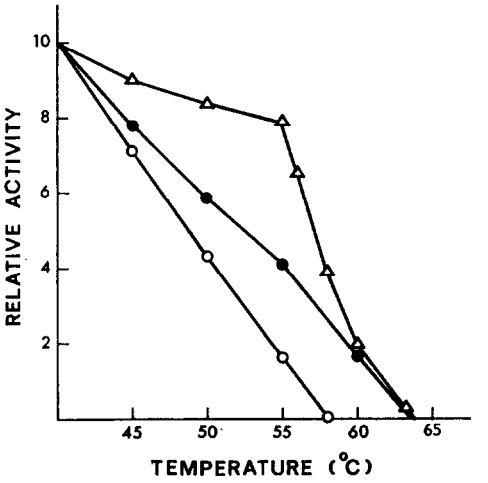

FIG. 6. TEMPERATURE INACTIVATION OF FUNGAL AND AVOCADO POLYGALACTURONASES.
△—△, Enzyme preparation of infected tissue; ○—○, enzyme preparation of uninvaded tissue; ●—●, fungal enzyme from sterilized avocado culture.

and then immediately cooled and assayed for activity. Linear inactivation curves were obtained with enzyme preparations of the uninvaded tissue of inoculated fruit and the fungal enzyme. This linear pattern of temperature inactivation indicates a single polygalacturonase. The fungal enzyme was more stable to temperature inactivation than the avocado enzyme. The sharp break in relative activity of the polygalacturonase from infected tissues (Fig. 6) clearly indicates that it contains two enzyme components. The fungal enzyme appears to have a higher affinity towards pectic acid than the avocado enzyme. The K_m values for fungal and avocado polygalacturonases were 0·051 per cent and 0·083 per cent, respectively. The maximal velocity was, however, higher with the avocado enzyme.

DISCUSSION

Based on the pattern of action on pectic acid (Fig. 5) and higher activity towards non-methylated substrates, the fungal enzyme can be identified as an endo-polygalacturonase. A fall in the noted pH of trigalacturonic acid cleavage and lower activity towards oligalacturonides were reported for other purified endopolygalacturonases.[12-14]

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[13] A. L. DEMAIN and H. J. PHAFF, *J. Biol. Chem.* **210**, 381 (1954).
[14] P. J. MILL and R. TUTTOBELLO, *Biochem. J.* **19**, 57 (1961).

The avocado polygalacturonase was also classified as a member of the endopolygalacturonases.[8] Although both the avocado and the fungal enzyme exhibit a similar pH optima of 5·5 with pectic or polygalacturonic acid, they differ in other ways. The avocado endopolygalacturonase produces a slow but distinct hydrolysis of digalacturonic acid and degrades pectic acid completely to D-galacturonic acid.[8] By contrast, the fungal endopolygalacturonase is incapable of attacking digalacturonic acid, and the accumulation of monogalacturonic acid during pectic acid hydrolysis reaches only 9 per cent of the total galacturonide bonds split. The avocado enzyme is more sensitive to temperature inactivation (Fig. 6) and has a lower affinity but higher maximal reaction velocity with pectic acid as compared to the fungal enzyme. The two enzymes also showed different electrophoretic mobility and adsorption properties on CM-cellulose.

The observation that fungal endopolygalacturonase constitutes only 16 per cent of the total polygalacturonase activity in the invaded tissue suggests that the avocado enzyme plays a major role in tissue maceration during anthracnose rot development. Furthermore, the appearance of avocado endopolygalacturonase is substantially induced in healthy parts of inoculated fruits (Fig. 1) while the fungal enzyme is limited to the infected areas. Thus the fruit seems to be predisposed by the pathogen for further invasion by stimulation of host polygalacturonase and tissue degradation.

The avocado fruit has the distinctive feature of remaining firm as long as the stem of the fruit is attached to the tree. After picking, it undergoes a ripening period with an upsurge in respiratory activity at the end of the maturation phase.[15] This phenomenon is known as the 'climacteric rise" in respiration and it is followed by final degradation of the fruit tissue. The protoplasmic disorganization during the phase of senescence is associated with pectin breakdown and endopolygalacturonase activity. The "climacteric rise" may be affected by a controlling balance between pectic enzymes, ethylene, auxin and other growth-regulating substances.[15] It thus appears that the invaded fungus has the capacity to trigger processes causing a rapid ripening and senescence. Ethylene, which is a common metabolic product of fungi[16] and diseased or injured plants,[17] may be involved in the above phenomenon. However, we have observed that neither injured nor sound fruits, which were kept for several days with infected fruits in a closed container, showed as marked a senescence as the infected avocados. It is, therefore, possible that factors other than ethylene may be involved in the mechanism of senescence induction by the pathogen.

It is not known whether the development of avocado endopolygalacturonase with ripening is the result of *de novo* synthesis of the enzyme or enzyme activation. Enzyme activation may occur by removal of an inhibitor present in the tissue or changes in enzyme conformation from an inactive to an active form. Although immature avocados were shown to contain a polygalacturonase inhibitor,[8] it is not clear whether or not it remains active upon maturation. Richmond and Bial[18] detected protein synthesis during the early stage of climacteric rise in avocado fruit. Thus the possibility that enzymes, which catalyze the final breakdown of the cell, are synthesized during this period, should be investigated. Avocados affected by anthracnose provide a suitable model for studying the control of endopolygalacturonase activity during the ripening period.

[15] J. B. Biale, *Science* **146**, 1 (1964).
[16] J. B. Biale, *Science* **91**, 458 (1940).
[17] S. P. Burg, *Ann. Rev. Plant Physiol.* **13**, 265 (1962).
[18] A. Richmond and J. B. Bial, *Arch. Biochem. Biophys.* **115**, 211 (1966).

EXPERIMENTAL

Method of Inoculation

A pathogenic strain of *Colletotrichum gloeosporioides* was isolated from infected avocado leaves and maintained in avocado agar[9] at 25°. Avocados of the Fuerte variety were used for inoculation tests immediately after harvesting. The fruits were surface-sterilized with 70% ethanol and a window-like section (1cm² × 2 mm thick) was cut and slightly raised above the peel surface. Fungal spores were then inserted beneath the sectioned peel and the wound was covered with sterilized paraffin. When fruits were held at 25° necrotic lesions appeared within 2–3 days. The lesions developed as black spots surrounding the inoculation site and reached a diameter of about 2·5 cm after 10 days. The fungus penetrated into the flesh of the fruits to a depth of approximately 1 cm and the invaded tissue became discolored. Samples of uninvaded tissue were taken for extraction at a distance of at least 10 cm from the discolored spot. Lesion appearance was immediately followed by softening in almost all parts of the fruits: in control (uninoculated) there was only very slight softening after 7 days.

Polygalacturonase Assay

Di- and trigalacturonic acid were prepared according to Hasegawa and Nagel[19] and were further purified using the procedure of Phaff and Luh.[20] Determination of average degree of polymerization (DP) has been described previously.[12]

The oligouronides produced single spots after paper chromatography and had DP values of 2·04 and 2·97 for the dimer and trimer, respectively. Sodium polypectate, pectic acid (Nutritional Biochemical Corp., Cleveland, Ohio) and polygalacturonic acid (Schuchardt, München) had an anhydrogalacturonic acid content[21] of 62%, 84% and 92%, respectively. Methyl esters of pectic and polygalacturonic acids were prepared according to McCready and Seegmiller.[22] Analyses showed that 52% of the carboxyl groups were esterified with methanol. All substrates were of analytical grade.

Unless otherwise stated the enzymatic reaction mixture contained 0·25% pectic acid, 0·1–0·5 ml enzyme preparation, 50 μmoles acetate buffer, pH 5·5, and water to make a total volume of 1 ml and was carried out at 30°. A reaction mixture of heat-denatured enzyme served as a control. Enzyme activity was determined by measuring reducing group liberation with a dinitrosalycilic acid (DNS) reagent[23] using galacturonic acid as a standard. The colorimetric method of Somogyi,[24] as modified by Patel and Phaff,[5] was used for measuring the rate of hydrolysis of oligogalacturonides. The appearance of free galacturonic acid was determined by a modified naphthoresorcinol procedure.[25] One unit of polygalacturonase was defined as being the amount of enzyme catalyzing the release of 1 mg galacturonic acid per hour under the above conditions. Viscometric measurements for polygalacturonase have been previously described.[11]

Extraction and Fractionation Procedures

For polygalacturonase extraction, the fruit part was cut into slices and suspended (1:1, w/v) in ice-cold 0·1 M acetate buffer, pH 6·5, containing 6% NaCl. The suspension was homogenized in a blender for 2 min and immediately cooled to 4°. The resulting homogenate was ground with one part (v/v) of acid-washed sand in a chilled mortar. The ice-cold buffer was added in small increments and the grinding was continued until 5 parts (v/v) of buffer had been added. The suspension was filtered through four layers of cheese-cloth and centrifuged at 10,000 *g* for 20 min. Lipids, which constitute the upper layer of the supernatant, were removed by suction. The supernatant was dialyzed for 3–4 days against water at 4° and was used as a crude enzyme preparation.

Cellulose *N,N*-diethylaminoethyl ether (DEAE) was obtained from Whatman and prepared according to Reymond and Phaff.[8] The cellulose was packed into a column (1·5 × 20 cm) and washed with 100 ml distilled water. The enzyme preparation was then placed on the column and washed with 100 ml of 0·005 M phosphate buffer, pH 8. Enzyme elution was carried out by a gradual increase in buffer strength and decrease in pH.[8] Carboxymethylcellulose (CMC) columns were prepared as described elsewhere[11] and equilibrated with 0·05 M acetate buffer of pH 4·5. Enzyme elution was performed by gradient increase in NaCl concentration from 0 to 0·5 M. All purification procedures took place at 4°.

[19] S. HASEGAWA and C. W. NAGEL, *J. Food Sci.* **31**, 834 (1966).
[20] H. J. PHAFF and B. S. LUH, *Arch. Biochem. Biophys.* **36**, 231 (1952).
[21] E. A. MCCOMB and R. M. MCCREADY, *Anal. Chem.* **24**, 1630 (1954).
[22] R. M. MCCREADY and G. C. SEEGMILLER, *Arch. Biochem. Biophys.* **50**, 440 (1954).
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[24] M. SOMOGYI, *J. Biol. Chem.* **160**, 61 (1945).
[25] M. B. RAHMAN and M. A. JOSLYN, *Food Res.* **18**, 308 (1953).

The method of Ornstein and Davis[26] was used for disc electrophoresis on polyacrylamide gel. Tris (hydroxymethyl)aminomethane-EDTA-boric acid buffer, pH 8·3, was used with a current regulated to 4 mA/tube. Corresponding gels were cut into sections of 0·125 cm each and tested for polygalacturonase activity.[12] Amount of protein was determined by the method of Lowry *et al.*[27] using bovine serum albumin fraction V as a standard.

[26] L. ORNSTEIN and B. J. DAVIS, Disc Electrophoresis Distillation Products Industry, Rochester, New York (1962).
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