Pectinases in Leaf Degradation by Aquatic Hyphomycetes: the Enzymes and Leaf Maceration

By ANNE-CAROLE CHAMIER* AND PETER A. DIXON

Department of Botany, Royal Holloway College, Egham, Surrey, U.K.

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All seven species of aquatic hyphomycetes tested produced both polygalacturonases and pectin lyases. The polygalacturonases were constitutive, whereas the pectin lyases were induced on pectic substrates at pH 6.5 and above. Some species could grow on pectic substrates at both pH 5 and pH 7; other species at pH 7 only. *Tricladium splendens* grew as well on a polypectate substrate as it did on glucose. This fungus elaborated three endo-polygalacturonase isoenzymes, as did *Articulospora tetraccladia*. *Tetrachaetum elegans* secreted a presumed exo-pectin lyase and a pectin esterase, and *Mycocentrospora angulata* an endo-pectin lyase and a pectin esterase. The four species macerated alder-leaf strips totally within 9 to 12 d at stream pH 7, utilizing predominantly pectin lyases and pectin esterases. These enzymes are stimulated by Ca^{2+} , and this may explain why plant decay proceeds most rapidly in calcium-rich streams of pH 6.5 and above. The frequency with which the four species of aquatic hyphomycetes were observed on experimental leaf packs placed in a stream does not appear to be related to their pectolytic capability.

INTRODUCTION

The degradation of allochthonous litter in freshwater streams has been associated with fungi and bacteria. Of the fungi found on decaying leaves, it seems likely that aquatic hyphomycetes are the most active in degrading leaf tissue in the stream environment (Triska, 1970; Kaushik & Hynes, 1971; Bärlocher & Kendrick, 1974; Suberkropp & Klug, 1976). But proof is required that these fungi are physiologically capable of breaking down and utilizing the complex polysaccharides of plant cell walls. It is possible that they draw only on leached substances as an energy source, or that they use the leaves as a holdfast and derive all their nutrients from the surrounding water, or that they live off the excess metabolites of other microbes. Proof of polysaccharidase capability in aquatic hyphomycetes grown on model and natural substrates would provide strong evidence that these organisms are active in degrading allochthonous litter.

The investigation of pectolytic capability is a logical first step, not only because pectic polysaccharides constitute the major component of primary cell walls (about 34%, w/w), but because pectic substances are the most immediately available polymers in non-lignified plant tissue and their degradation exposes the other components, the hemicelluloses (about 24%) and cellulose (about 19%) for enzymic attack (Darvill *et al.*, 1980).

The aim of this study was to investigate the pectolytic capability of some aquatic hyphomycetes and to ascertain whether their pattern of colonization of experimental leaf-packs in a stream (Chamier & Dixon, 1982) reflects their capacity to degrade pectic polysaccharides of leaf cell-walls.

METHODS

Fungi and culture media. The following species of aquatic hyphomycetes were isolated from scum samples taken from the River Bourne (Chamier & Dixon, 1982): *Articulospora tetraccladia* Ingold, *Lemonniera aquatica* de Wild.,

Abbreviations: GalUA, galacturonic acid; UDGalUA, unsaturated digalacturonic acid; Napp, sodium polypectate; PE, pectinesterase; PG, polygalacturonase; PGL, polygalacturonate lyase; PL, pectin lyase; RVU, 1/(time to 50% viscosity); TBA, thiobarbituric acid.

Mycocentrospora angulata (Petersen) Iqbal, *Tetrachaetum elegans* Ingold, *Tetracladium setigerum* (Grove) Ingold, *Tricladium splendens* Ingold and *Varicosporium elodeae* Kegel. The fungi were grown at 15 °C on the following media. (a) Plate medium GYS (L. G. Willoughby, personal communication) (g l⁻¹): glucose, 10; soluble starch, 5; yeast extract, 2; Na₂HPO₄ · 12H₂O, 0.6; KH₂PO₄, 2.04; agar, 20. (b) Flask medium. As above, without glucose, starch and agar but with the appropriate carbon source (1%, w/v): sodium polypectate (Napp; Nutritional Biochemicals, Cleveland, Ohio, U.S.A.; Batch 2837); galacturonic acid (GalUA; BDH); pectin (apple, 250 grade; BDH). (c) Pectinase assay plates (Hankin & Anagnostakis, 1975). The full medium was used for solid medium culture experiments. Cup-plate assays for polygalacturonase (EC 3.2.1.15; PG) activity were made on solid medium consisting of 11 McIlvane's buffer (0.15 M, pH 5.0), 5 g Napp and 15 g agar. After autoclaving at 121 °C for 15 min, the medium was poured into sterile Petri dishes (25 ml per plate). Wells (1 cm diam.) were cut in the gel with a flamed no. 5 cork-borer and filled with enzyme sample. The plates were incubated for 18 h at 30 °C then flooded with 1% (w/v) hexadecyltrimethylammonium bromide to precipitate undegraded Napp. A clear zone around the well indicates PG activity.

Cup-plates for assays of pectin lyase (EC 4.2.2.10; PL) activity were made in the same way on medium consisting of 1 l Tris/HCl buffer (0.2 M, pH 9.0), 5 g pectin and 15 g agar. After incubation for 18 h at 30 °C, the plates were flooded with 5 M-HCl. A precipitated, white zone around the well indicates PL activity and a clear zone, pectinesterase (EC 3.1.1.11; PE) activity.

Enzyme activity is expressed as the radius of the ring around the well and does not include the well. The radius of the ring is approximately proportional to the logarithm of enzyme activity (Archer, 1979). PG activity was detected on Napp plates at pH 5 only, and not at pH 7 or on pectin plates at pH 9. PL activity was not detected on Napp plates at pH 5 or pH 9, but showed some activity on Napp plates at pH 7, though this was not as marked as activity on pectin plates at pH 9. PE activity was not detected on any Napp plates.

Centrifugation. This was carried out under refrigeration in an MSE Superspeed 40 (8 × 25 ml) at 7000 g for 20 min or in an MSE Superspeed 65 (6 × 300 ml) at 45000 g for 2 h.

Washed Napp and pectin. To remove low molecular weight contaminants, Napp and pectin were washed firstly in acidified (0.05 M-HCl) 70% (v/v) ethanol, then in 95% ethanol and finally in absolute ethanol (Cooper & Wood, 1975).

Viscometry. Loss of viscosity in pectic reaction mixtures was measured in a Cannon-Fenske size 200 viscometer suspended in a water bath at 30 °C. Viscosity-loss was calculated from the following equation: $[(V_0 - V_t)/(V_0 - V_s)] \times 100$, where V_0 = flow-time (s) at t_0 ; V_t = flow-time at subsequent times; V_s = flow-time of 10 ml distilled water, i.e. 100% viscosity loss.

For PG, substrate solutions containing 1% (w/v) washed Napp were made up in 0.075 M-McIlvaine's buffer to cover the range pH 4.0–7.0. Reaction mixtures were 9 ml substrate and 0.5 ml or 0.1 ml enzyme. The reciprocal of the time (min) taken to achieve a 50% reduction in viscosity was designated RVU (relative viscosity units) of enzyme activity. For PL activity the substrate was a 0.6% (w/v) solution of washed pectin made up with 1 mM-CaCl₂ to pH 9.0 in 0.025 M-Tris/HCl buffer.

Dry weight measurements. These were made on pre-dried, pre-weighed Whatman glass-fibre papers. Mycelium, resuspended in water after separation from culture filtrates by centrifugation, was filtered on to the papers under vacuum. Papers plus mycelium were dried for 18 h at 90 °C, cooled in a desiccator and reweighed.

Pectinase induction, purification and characterization. These were carried out according to Fanelli *et al.* (1978).

(a) **Enzyme induction.** Flask medium (2 l) was made up in 0.1 M citrate buffer, pH 5.0 for PG induction and in medium adjusted to pH 7.0 for PL induction. 1 mM-CaCl₂ was added to the PL medium. The pH 5.0 medium requires strong buffering, otherwise fungal metabolism raises the pH, which stimulates the production of PL rather than PG. Napp (1%, w/v) was added to the medium and dissolved over low heat. The medium was then distributed between eight 1 l conical flasks, which were then stoppered with cotton wool and autoclaved at 121 °C for 15 min. To the cool, sterile medium was added 20 ml spore inoculum (10⁴ spores ml⁻¹). The culture was grown on a refrigerated Gallenkamp orbital shaker (90 rev. min⁻¹) at 15 °C for 11 d, after which the mycelium was filtered off with butter muslin and the culture filtrate centrifuged. The culture filtrates for PG had a final pH of about 5.7, those for PL a final pH of about 8.0. Enzyme samples were stored at -12 °C, thawed and maintained on ice.

(b) **Enzyme purification.** Ultrafiltration, dialysis and ion-exchange chromatography were carried out at 10 °C. (i) Ultrafiltration. An Amicon stirred ultrafiltration cell (model 402) with reservoir was used with Amicon UM10 or PM10 Diaflo ultrafiltration membranes. The sample was concentrated from 1500 ml to about 150 ml.

(ii) **Dialysis.** The ultrafiltrate was dialysed extensively against the column starting buffer.

(iii) **Ion-exchange chromatography.** The ion-exchangers used were CM-Sepharose CL-6B and DEAE-Sepharose CL-6B (Pharmacia), packed into 130 ml gel beds. The starting buffers were 0.02 M-acetate at pH 5.0 or 4.0 for cation exchange, and 0.025 M-Tris/HCl for anion exchange at pH 7.2. Flow-rate was 15 ml h⁻¹. Fractions of 5 ml were eluted with a linear NaCl gradient (400 ml) from 0 to 1 M. Fractions were collected until the salt gradient was completed and no protein was detected for at least 3 h. Cup-plate assays were carried out on every second fraction. Fractions with high enzyme activity were pooled and stored at -12 °C.

(iv) Polyacrylamide gel electrofocusing. Polyacrylamide gel plates (LKB, pH 3.5–9.5) were used to obtain an approximate pI value for PGs, on an LKB Multiphor 2117 maintained at 6 °C. After replicate samples had been subject to electrofocusing, the gel was cut into strips, which were placed on assay plates and incubated at 30 °C for 18 h. Enzyme activity could thus be detected and an approximate value for pI obtained from a graph of the pH gradient against gel width. Electrofocusing was found to be unsuitable for PL. The ampholines reacted with pectin to give precipitates similar to those caused by enzyme activity. Napp plates gave no reaction to PL activity at pH 9 and were unreliable at pH 7.

(v) Preparative iso-electric focusing. The preparative gel used was LKB Ultradex 510 with an LKB Multiphor 2117 cooled by a constant flow of tap water. pI was taken as the pH range in which enzyme activity was detected in fractions eluted from gel strips. Enzyme fractions were pooled and stored at –12 °C.

(vi) Protein estimation. The Lowry method was used, with bovine serum albumin as the standard.

(c) PG characterization. (i) pH optimum was established by viscometry.

(ii) Reducing-group analysis for K_m , V_{max} and percentage hydrolysis of substrate at 50% viscosity loss. Hydrolysis of glycosidic bonds by enzyme activity was monitored by the reducing-groups assay, using the dinitrosalicylic acid method of Miller (1959). The standard curve and all readings were made against a reagent blank on hot samples, as cooling caused gelling of residual Napp. A reducing-group value for complete enzymic degradation of a 1% (w/v) solution of Napp was made with 9 ml substrate incubated with 1 ml of a 1% (w/v) solution of commercial PG, at 30 °C for 24 h.

(iii) Paper chromatography. The products arising from the action of each PG on a Napp substrate were analysed by descending paper chromatography (Whatman no. 4 paper), by the method of Nasuno & Starr (1966). The standard was 75 mM-GalUA. The reaction mixture (9 ml) was as used for viscometry, incubated at 30 °C at optimal pH. Samples (25 µl) were taken at intervals up to 24 h from the reaction mixture and directly spotted on to the paper. The solvent (1-butanol/acetic acid/water, 4:2:3, by vol.) was run to the bottom of the paper which was then dried overnight. Uronic acids were detected by spraying the paper with 0.04% (w/v) bromophenol blue in 95% (v/v) ethanol adjusted to pH 7.0. Only acidic substances are visualized, and appear yellow on a blue ground. Oligomers were identified from their R_{GalUA} values, given by Nasuno & Starr (1966). The limits of detectability of uronic acids was about 8.5 µg.

(d) PL characterization. (i) Thiobarbituric acid (TBA) assay for pH optimum and K_m (Quantick, 1978, after Ayers *et al.*, 1966). To determine the pH optima for PL, solutions of washed pectin, 1% (w/v), were made up in 0.025 M-Tris/HCl buffer ranging from pH 7.2–9.8. Reaction mixtures consisted of 4 ml pectin/buffer solution with 2 ml enzyme (*Tetrachaetum elegans*) or 5 ml pectin/buffer solution with 1 ml enzyme (*Mycocentrospora angulata*). The final concentration of Ca^{2+} was 1 mM. Reaction mixtures were incubated for exactly 1 h in a water bath at 30 °C. A sample (1 ml) was added to 5 ml TBA solution (0.04 M) and 2.5 ml HCl (1 M) in a test tube and mixed well. The tube was covered with a metal cap and placed in a boiling water bath for 30 min. Before cooling, the absorbance of the mixture was read against a reagent blank at 550 nm on a Unicam Sp 500 spectrophotometer. Cooling resulted in clouding of the mixture. A standard curve was prepared using 4-O- α -D(4,5-dehydrogalacturonosyl)-D-galacturonic acid (unsaturated digalacturonic acid, UDGalUA; kindly given by Professor R. H. Vaughn, University of California, Davis). Enzyme activity was expressed as release of UDGalUA. Because pectin hydrolyses spontaneously in neutral-to-alkaline conditions, substrate alone was incubated at the same time and temperature as the reaction mixture and assayed so that a true value for enzymic degradation could be calculated. A value for total hydrolysis of bonds to give UDGalUA was made with a PL derived from *Mycocentrospora angulata*. The reaction mixture (as above) plus 0.1% (w/v) phenol was incubated at 30 °C for 7 d then assayed.

(ii) Paper chromatography. The method given for PG was used. UDGalUA was the standard and the unreacted substrate was sampled at the end of incubation so that spontaneous hydrolysis would be accounted for.

(e) Pectin esterase characterization. The assay used was that described by Delincée & Radola (1970). Enzyme (6 ml) was added to an unbuffered solution (20 ml) of washed pectin (1%, w/v) adjusted to pH 9.0. The pH of the mixture and this substrate alone were measured at fixed time intervals at 30 °C. Changes of pH due to spontaneous de-esterification of the pectin are thus taken into account in the calculation of enzyme activity given as $\Delta pH (mg \text{ protein})^{-1} \text{ min}^{-1}$.

Leaf maceration. Entire, abscised leaves of alder (*Alnus glutinosa*) were collected and air-dried at room temperature. Before use, they were soaked in tepid water for 2 h. Two identical leaf strips were cut around a copper template 4.5 × 2 cm, from either side of the midrib of the lamina. Matching strips were notched for identification and surface-sterilized with propylene oxide.

To 1 l of stream water were added the following mineral salts (g): KNO_3 , 1.0; KH_2PO_4 , 1.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.1. The solution was unbuffered and adjusted to the pH of the stream water, about pH 7. The solution was sterilized by membrane filtration (0.45 µm) and samples (35 ml) were pipetted into sterile, matched 150 ml conical flasks. Flasks were paired, and three sterile leaf strips were placed in one flask and the matching pairs of leaf strips in the other. One flask of each pair was uninoculated; into the other was pipetted 2×10^4 spores (2 ml suspension). The flasks were placed in pairs on a Gallenkamp refrigerated orbital shaker (90 rev. min^{-1}) at 15 °C. The tensile strength of the pairs of leaf strips was measured every alternate day of

incubation on an Instron tensiometer. The tensile strength of the inoculated strip is expressed as a percentage of that of the matching control strip. Undegraded matched pairs always had a tensile strength within 10% of each other, so a decrease in tensile strength below 80% of the matching control was considered significant evidence of leaf maceration by fungal activity. Sampling was carried out until inoculated leaves had less than 5% tensile strength of the controls, or had disintegrated. On the day of sampling, the pH of the supernatant fluids was measured and the fluids assayed for pectolytic activity by cup-plate methods.

RESULTS AND DISCUSSION

Growth and pectinase production by Tricladium splendens

This experiment compared growth and pectinase production by *Tricladium splendens* on a glucose substrate with that on pectic substrates. Flask medium (25 ml in 100 ml conical flasks) at pH 5 containing 1% (w/v) each of glucose, Napp/GalUA (1:1), or Napp alone, was inoculated with 2 ml spore suspension. After 5 d at 15 °C, three flasks of each carbon source were sampled daily. The dry weight of mycelium and the pH of the culture filtrate were measured. The percentage viscosity loss of the pectic medium was calculated and pectinase activity was assayed on cup-plates at pH 5 (PG) and pH 9 (PL).

Tricladium splendens grew as well on a polypectate substrate as on a monomer, glucose (Fig. 1). Lower growth rates on Napp/GalUA may be attributable to the lower initial pH of 4.3. The production of PG on a glucose substrate indicates that this enzyme is constitutive. PG activity on pectic substrates was substantially greater, indicating that the presence of a specific substrate stimulates production of the constitutive enzyme. No PL activity was detectable on the glucose substrate and there was little change in the pH of the culture filtrate. In the pectic media there was a marked rise in pH and at pH 6.5, PL activity appeared.

In similar experiments with six other species of aquatic hyphomycetes investigated (Chamier, 1980), PG was always detectable in culture filtrates from about pH 5 to about pH 8, on both glucose and pectic substrates, though levels were low on glucose and at pH 6.5 and above. PG is evidently constitutively produced by the species investigated. PL was not detected on a glucose substrate whatever the pH and appeared only on pectic substrates where the pH was 6.5 and above. This suggests that PL is induced in the presence of its substrate and that its production is pH dependent.

Pectinase production and growth of aquatic hyphomycetes on solid Napp medium

This experiment was designed to test the capacity for growth and pectinase production by aquatic hyphomycetes at pH values corresponding to acidotrophic waters (pH 5) and harmonic waters (pH 7). Discs (10 mm diam.) were cut from fungal colonies growing on GYS agar plates. One disc was placed in the centre of each Napp assay plate, which was incubated at 15 °C for 11 d, then developed with hexadecyltrimethylammonium bromide.

All species tested could produce both PG and PL (Table 1). *Tricladium splendens* and *Varicosporium elodeae* grew as well on a pectic substrate at pH 5 as at pH 7. *Lemonniera aquatica* and *Mycocentrospora angulata* grew very little and *Tetracladium setigerum* and *Tetrachaetum elegans* not at all at pH 5, though at this pH these species produced PG constitutively. This was confirmed by the production of PG, but not PL, by *Tetracladium setigerum* on GYS medium at pH 5. The extent of pectinase activity varied between species, and the amount of growth was not related to the extent of pectinase activity. The differences observed between species show that each has an individual degree of physiological adaptation to acidic and neutral-to-alkaline conditions, which may be based on enzymic capability and on optimum pH for pectinase activity, but also involves factors other than pectolytic capability.

From the results of these and further preliminary experiments (Chamier, 1980), four species were chosen for large-scale production of pectinases so that these could be separated, purified and characterized. *Tricladium splendens*, which was rare on experimental leaf-packs placed in a stream (Chamier & Dixon, 1982) and *Articulospora tetracladia*, which appeared more often, were chosen for PG production. *Tetrachaetum elegans*, the dominant species on leaf-packs, and *Mycocentrospora angulata*, another persistent colonizer, were chosen for PL production.

Fig. 1. *Tricladium splendens* grown on three carbon sources each at 1% (w/v). ●, Dry weight of mycelium; ○, pH of culture filtrate. The PG assay was done on cup-plates at pH 5.0, and the PL assay on cup-plates at pH 9.0. The results of the cup-plate assays are expressed as the radius of the ring around the well, in mm from the well margin (✓, activity present but not quantified; ×, activity absent). Percentage decrease in initial viscosity with time is shown.

Table 1. *Pectinase production and growth of aquatic hyphomycetes on solid Napp medium at pH 5 and pH 7*

The radii of enzyme activity were measured from the colony margin.

Species	pH 5 plates		pH 7 plates	
	Colony diameter* (mm)	Radius of PG activity (mm)	Colony diameter* (mm)	Radius of PL activity (mm)
<i>Tricladium splendens</i>	25	22	25	5
<i>Varicosporium elodeae</i>	36	2	38	3
<i>Articulospora tetracledia</i>	16	3	25	3
<i>Lemonniera aquatica</i>	12	20	16	10
<i>Mycocentrospora angulata</i>	13	5	23	5
<i>Tetracledium setigerum</i>	10	8	33	2
<i>Tetrachaetum elegans</i>	10	15	23	10

* Includes original 10 mm core. All replicates gave identical results.

Polygalacturonases of Tricladium splendens

For enzyme purification, filtrate (1500 ml) from a *Tricladium splendens* culture grown in buffered flask medium, pH 5, containing 1% (w/v) Napp (final pH about 5.7) was centrifuged then ultrafiltered to 150 ml. PG, but not PL activity was present in the sample; neither was present in the effluent. The sample was dialysed for 24 h then applied to a cation-exchange column at pH 5.0. The column was eluted with a linear NaCl gradient and every alternate 5 ml fraction assayed by cup-plate for PG activity. The elution profile (Fig. 2a) shows three separate zones of PG activity, designated enzymes I, II and III. As enzyme I was not adsorbed on to the column, it was rechromatographed on the same exchanger equilibrated to pH 4.0. The elution profile (Fig. 2b) shows that enzyme I was adsorbed and separated from most of the background protein in the sample.

Fig. 2. Elution profile of PG in culture filtrate of *Tricladium splendens* grown in buffered flask medium, pH 5.0, containing 1% (w/v) Napp (final pH about 5.7). (a) Separation carried out on a cation-exchange column of CM-Sephacrose buffered with 0.02 M-acetate at pH 5.0. (b) Fractions 16-37 from (a) run on the same column buffered to pH 4.0. The flow-rate for elution (a, b) was 15 ml h⁻¹. Fractions (5 ml) were assayed by cup-plate for PG activity (●). —, Protein concentration (transmittance, T_{280}); ····, NaCl concentration. I, II and III indicate the positions of the three PG enzymes.

Table 2. Purification and properties of three polygalacturonases produced by *Tricladium splendens*

	Culture filtrate	Enzyme I	Enzyme II	Enzyme III
Volume (ml)	1500	6	6	20
Protein (mg ml ⁻¹)	0.16	1.2	1.8	0.26
pH optimum	—	5.25	5.0	5.6
Hydrolysis at 50% viscosity loss (%)	—	1.8	2.0	4.0
Isoelectric point	—	8.4 ± 0.8	5.2 ± 0.2	7.3 ± 0.2
K_m (mg ml ⁻¹)	—	4.3	2.5	1.34
V_{max} [μ g GalUA min ⁻¹ (mg protein) ⁻¹]	—	23.8	277.7	296.2

Approximate pI values for enzymes I, II and III were obtained by electrofocusing on polyacrylamide gel. Narrow-range ampholines, covering the range of the approximate pI values of each enzyme, were used for purification by preparative gel iso-electric focusing. Accurate pI values were obtained by this method (Table 2).

The pH optimum of each enzyme was obtained by viscometry (Fig. 3d, e, f). Percentage viscosity loss of the substrate at optimum pH and percentage hydrolysis of the substrate at 50% viscosity loss for enzymes I, II and III are shown in Fig. 3 (a, b, c). K_m and V_{max} values (Table 2) were calculated by the Lineweaver-Burk equation. Values for V_{max} are based on mg enzyme protein in the reaction mixture. Table 2 summarizes the data on the purification and properties of enzymes I, II and III. Enzyme I appears anomalous in comparison with enzymes II and III.

Fig. 3. (a, b, c) Relative viscosity decrease (●) and percentage hydrolysis of substrate (■) by enzymes I, II and III as a function of time at optimum pH; (d, e, f) pH optimum curves for enzymes I, II and III measured by viscometry. See Methods for definition of RVU. Reaction mixtures were 9 ml substrate [1% (w/v) solutions of Napp in buffer] and 0.5 ml enzymes I and III, or 0.1 ml enzyme II, incubated at 30 °C.

Theoretically, from its behaviour on ion-exchange chromatography, it should have a lower pI value than enzymes II and III, whereas it has a higher pI. This, together with the wide range of the pI value, suggests that the enzyme may have an unstable net charge. The $V_{\max}$ for enzyme I is low compared with the values obtained for the other PGs; though the 1% (w/v) Napp solution may not have been fully saturating to an enzyme of high K_m , producing an underestimation for $V_{\max}$, the discrepancy would not be great.

The reaction products of enzymes I, II and III were analysed by descending paper chromatography, to ascertain their mode of action. The enzymes have a similar action pattern, that of random hydrolysis of the pectate polymer. The presence in all three reaction mixtures of oligouronic acids from the pentamer to the monomer after 2 h (enzyme II), 4 h (enzyme III) and 7 h (enzyme I) is evidence of a generally random cleavage of glycosidic bonds in the polymeric substrate. The concentration of the higher molecular weight oligomers declined with time whilst that of the tri-, di- and monomeric acids increased. These results were similar to those produced by the same method for an endopolygalacturonase of *Rhizoctonia fragariae* (Cervone *et al.*, 1977) and two endopolygalacturonases of *Trichoderma koningii* (Fanelli *et al.*, 1978). The evidence provided by the chromatograms accords with the low percentage hydrolysis of bonds at 50% viscosity loss of the substrate. Enzymes I, II and III may be classified as endo-polygalacturonases: poly(1,4- α -D-galacturonide) glycanohydrolase.

Though the action pattern is generally of random cleavage of glycosidic bonds, it is not entirely so. The monomer was detectable in all cases after 30 min reaction, and its concentration increased with time in a way that could not be due to a simple random cleavage. To test whether this was due to monomer in the substrate, Chamier (1980) made a TCL analysis of the reaction products of enzyme I; the results confirmed those of the paper chromatography. There is no evidence of monomer in the unreacted substrate, nor after 6 min incubation, but after 30 min

Fig. 4. Elution profile of PG in culture filtrate of *Articulospora tetracladia* grown in buffered flask medium, pH 5.0, containing 1% (w/v) Napp (final pH about 5.7). (a) Separation carried out on a cation-exchange column of CM-Sephacrose buffered with 0.02 M-acetate at pH 5.0. (b) Fractions 14–50 from (a) run on the same column buffered to pH 4.0. The flow rate for elution (a, b) was 15 ml h⁻¹. Fractions (5 ml) were assayed by cup-plate for PG activity (●). —, Protein concentration (transmittance, T_{280}); ---, NaCl concentration. A, B and C indicate the positions of the three PG enzymes.

reaction, GalUA is detectable in the mixture. This means that a terminal residue must be cut off more regularly than random cleavage of bonds suggests, and that the designation 'endo'-PG does not strictly hold. Cooper & Wood (1975) made a similar observation on fungal PG.

Polygalacturonases of Articulospora tetracladia

The procedures used for the induction, separation, purification and characterization of PG of *Tricladium splendens* were used for *Articulospora tetracladia* grown in buffered flask medium, pH 5.0, containing 1% (w/v) Napp (final pH about 5.7). Cation-exchange chromatography elution profiles at pH 5.0 and 4.0 are given in Fig. 4 (a, b). Enzyme C was adsorbed on to the column at pH 5 and separated from enzymes A and B which were not adsorbed. Enzyme B was separated from enzyme A at pH 4 though enzyme A was not adsorbed. The three enzymes were further purified by preparative gel iso-electric focusing. pH optimum curves are given in Fig. 5 (d, e, f), and viscosity loss at optimum pH with percentage hydrolysis of the substrate at 50% viscosity loss in Fig. 5 (a, b, c). Lineweaver-Burk plots were used to calculate K_m and V_{max} for enzymes A, B and C. The purification and properties of these enzymes are summarized in Table 3.

The reaction products of enzymes A, B and C were analysed by descending paper chromatography. Enzymes A and B have a somewhat different action pattern from enzyme C. Enzyme C is an endo-PG with an action pattern very similar to those of *Tricladium splendens*, but with a stronger exo-element seen in lower concentrations and quicker breakdown of oligomers compared with the rapid increase in the concentration of the monomer. The evidence of predominantly random attack of the polymer by enzyme C is supported by the low percentage hydrolysis of the substrate at 50% viscosity loss and the swift reduction of viscosity. Enzymes A and B produce only monomer, dimer and trimer as reaction products. Of these, the dimer and monomer predominate as the enzymes degrade the trimer with time. Superficially, these are the

Fig. 5. (a, b, c) Relative viscosity decrease (●) and percentage hydrolysis of substrate (■) by enzymes A, B and C as a function of time at optimum pH; (d, e, f) pH optimum curves for enzymes A, B and C measured by viscometry. See Methods for definition of RVU. Reaction mixtures were 9 ml substrate [1% (w/v) solutions of Napp in buffer] and 0.5 ml enzyme, incubated at 30 °C.

Table 3. *Purification and properties of three polygalacturonases produced by Articulospora tetracladia*

See Fig. 4 for elution profiles of enzymes A, B and C.

	Culture filtrate	Enzyme A	Enzyme B	Enzyme C
Volume (ml)	1500	15	10	10
Protein (mg ml ⁻¹)	0.14	0.62	0.48	0.38
pH optimum	—	5.5	5.5	5.5
Hydrolysis at 50% viscosity loss (%)	—	3.5	3.0	2.5
Isoelectric point	—	4.4 ± 0.6	7.5 ± 0.1	8.0 ± 0.1
K_m (mg ml ⁻¹)	—	0.98	0.72	0.89
V_{max} [μ g GalUA min ⁻¹ (mg protein) ⁻¹]	—	383.9	198.4	198.3

characteristics of exo-enzymes, but with enzymes A and B, 50% viscosity loss of the substrate is swift and accompanied by a low percentage hydrolysis, both characteristics of endo-enzymes. The action pattern is unusual, but was recorded by English *et al.* (1972) for an endo-polygalacturonase produced by *Colletotrichum lindemuthianum*. They suggested that the enzyme initially attacked the substrate molecules at random. The attack then progressed from that site along the chain, releasing monomer, dimer and sometimes trimer until it reached a barrier such as a branch point or a neutral sugar residue; the enzyme would then attack another molecule. Evidence for a similar 'multiple attack' by glycosidases is given for amylases. In extreme cases the enzyme completely hydrolyses one substrate molecule before reacting with another (Thoma *et al.*, 1971). Enzymes A, B and C may be classified as endo-polygalacturonases.

Fig. 6. Elution profiles of culture filtrates of *Tetrachaetum elegans* and *Mycocentrospora angulata*. The flow-rate for elution (a, b, c) was 13.5 ml h^{-1} . Fractions (4.5 ml) were assayed by cup-plate for PL activity (●). —, Protein concentration (transmittance, T_{280}); ---, NaCl concentration. (a) *Tetrachaetum elegans* grown in buffered flask medium, pH 7.0, containing 1% (w/v) Napp (final pH 8.0). Separation carried out on a cation-exchange column of CM-Sepharose buffered with 0.02 M-acetate, pH 5.0. (b) *Mycocentrospora angulata* grown in buffered flask medium, pH 7.0, containing 1% (w/v) Napp (final pH 8.0). Separation carried out on an anion-exchange column of DEAE-Sepharose buffered to pH 7.2 with 0.025 M-Tris/HCl. (c) As in (b) using culture filtrate from *Tetrachaetum elegans*. The positions of PL α , PL β and PE are indicated.

Enzymes I, II and III of *Tricladium splendens* are isoenzymes, as are enzymes A, B and C of *Articulospora tetracladia*. The differences in properties between the isoenzymes within a species suggests that they are genetically independent proteins, the products of separate genes. If pH optima, K_m and V_{max} values for each isoenzyme complex are compared, the PG of *Tricladium splendens* would be active over a wider range of pH and substrate concentrations than those of *Articulospora tetracladia*, but at lower overall velocity. These differences are evident in the comparative performances of the two species on solid pectic medium at fixed pH 5.0 (Table 1).

Pectin lyases of Tetrachaetum elegans and Mycocentrospora angulata

The ultrafiltered, dialysed culture filtrate from *Tetrachaetum elegans* grown in buffered flask medium, pH 7.0, containing 1% (w/v) Napp and 1 mM- CaCl_2 (final pH about 8.0) was applied to a cation-exchange column at pH 5.0. Alternate fractions were assayed on pectin cup-plates

Fig. 7. pH optimum curve for PL α of *Tetrachaetum elegans* (a) and PL β , of *Mycocentrospora angulata* (b). Values represent percentage of maximum activity assayed by TBA method. Reaction mixture for (a) was 4 ml 1% (w/v) solution of pectin in buffer and 2.0 ml enzyme. Ca²⁺ concentration 1 mM. Reaction mixture for (b) was 5 ml of the same solution and 1.0 ml enzyme, incubated at 30 °C.

(pH 9) to detect PL activity. The elution profile (Fig. 6a) shows that the PL was not adsorbed on to the column, but a pectic enzyme was eluted in fractions 76–92 which gave a clear zone around the well of the assay plate. A TBA assay conducted on this enzyme using a 1% (w/v) solution of pectin at pH 9 (Ca²⁺ 1 mM) showed little increase in absorption with time at 510 nm or at 550 nm. The enzyme gave no reaction on Napp assay plates at pH 5 or pH 9. The PE assay gave a Δ pH of 0.5 (mg protein)⁻¹ min⁻¹ and, though this enzyme requires more thorough investigation, it is probably a pectin esterase. PEs of microbial origin often have pH optima of about pH 5, but a number with alkaline optima are recorded by Rexová-Benková & Markovič (1976), including a PE of *Clostridium multifementans* with an optimum of pH 9.0.

The PL sample was applied to a cation-exchange column at pH 4.0, but was not adsorbed. Isoelectric focusing was used in an attempt to purify the PL, but the ampholine carriers reacted with pectin and interfered with the assay, so this procedure was not used in the purification of PL. A fresh batch of ultrafiltered and dialysed enzyme was applied to an anion-exchange column equilibrated to pH 7.2. The elution profile of this separation is given in Fig. 6(c). The elution profile for a similar sample obtained from *Mycocentrospora angulata* is shown in Fig. 6(b). The PL α was that prepared from *Tetrachaetum elegans* and the PL β from *Mycocentrospora angulata*. Although some PG activity was detected in the unadsorbed protein fractions, no other pectic enzymes were detected in the pooled fractions containing PL.

pH optimum curves for PL α and PL β obtained by TBA assay are given in Fig. 7 (a, b). The pH optimum for both enzymes was 9.0 on the substrate and at the Ca²⁺ concentration (1 mM) used in these determinations. Rexová-Benková & Markovič (1976) reported lower pH optima for PL, depending on the degree of esterification of the substrate. With decreasing degree of esterification the pH optimum is lowered. PL α assayed under the same conditions and concentrations on a Napp substrate gave 33% of the pectin maximum and PL β , 52%. PL showed no reaction on Napp assay plates at pH 9, but some at pH 7, which suggests that its pH optimum on this substrate may be lower. Table 4 summarizes the data on the partial purification and properties of PL α and β .

A viscometric assay was carried out to establish the time taken for 50% viscosity loss and the percentage hydrolysis of the substrate at that point. For PL α , 8 ml of a 0.6% (w/v) solution of washed pectin and 2 ml enzyme (final Ca²⁺ concentration of 1 mM, pH 9.0) were used, and for PL β , 9 ml substrate and 1 ml enzyme. Both were incubated at 30 °C. PL α gave 41% hydrolysis of the substrate at 50% viscosity loss and PL β , 3%.

The reaction products of these enzymes were analysed by descending paper chromatography. The reaction mixtures were those given for TBA assays with 0.1% (w/v) phenol as a microbial inhibitor. PL α was incubated for 55 h and PL β for 48 h at 30 °C. PL α appears to be an exo-enzyme, as no intermediate products between UDGalUA and the polymer were detected in 55 h of incubation; PL β is an endo-enzyme, since oligomers were detected after 24 h incubation and

Table 4. *Data on partial purification and properties of PL α and PL β , from Tetrachaetum elegans and Mycocentrospora angulata, respectively*

Both fungi were grown in buffered flask medium, pH 7.0, containing 1% (w/v) Napp (final pH about 8.0).

Organism	Initial volume (ml)	Total protein (mg)	Volume after ultra-filtration (ml)	PL	Final volume (ml)	Protein (mg ml ⁻¹)	pH optimum	K _m (mg ml ⁻¹)
<i>Tetrachaetum elegans</i>	1500	220	150	PL α	30	0.7	9.0	1.1
<i>Mycocentrospora angulata</i>	1500	210	150	PL β	32	0.65	9.0	4.0

the intensity of their concentration increased with time. Although esters would be formed by the action of lyases on pectin, only acidic substances would be visualized on the chromatogram by the method used. The early appearance of UDGalUA in the products can be ascribed to spontaneous hydrolysis of the substrate at alkaline pH, but the observed increase in concentration with time beyond the levels detectable in the unreacted substrate blank is the result of enzymic degradation of the pectin. Some streaking of the sample in PL α at 31 and 55 h probably represents shortened substrate chains that have not been reduced to detectable oligomers.

Although there is no official recognition of exo-pectin lyase in the classification of enzymes, Cooper (1974) reported a PL with this action pattern in *Verticillium albo-atrum*. The evidence from chromatography is supported by the results of the viscometric assay. Exo-enzymes, cleaving only terminal bonds, are slow to reduce the viscosity of the substrate with about 40% hydrolysis of bonds at 50% viscosity loss. Endo-enzymes, cleaving bonds at random, produce a much swifter reduction in substrate viscosity and a 50% loss is associated with about 2% hydrolysis of available bonds.

PL β can be classified as a pectin lyase: poly(methoxygalacturonide) lyase. This enzyme is defined as having a random action pattern on its substrate. There are no official records for a PL with a terminal action pattern.

Leaf maceration by aquatic hyphomycetes

Keegstra *et al.* (1972) reported that purified polysaccharidases of *Colletotrichum lindemuthianum* were unable to degrade isolated host cell walls. To test whether pectinases, which are expressed by the organisms *in vitro* on model substrates, are active when the organisms are grown on one of the leaf species used in field experiments (Chamier & Dixon, 1982), experiments measuring maceration of alder leaves at stream pH were conducted. Changes in the tensile strength of inoculated leaves with time, as a percentage of the value for uninoculated leaves, are shown in Fig. 8 (*a, b*). The initial pH of the medium was that of the stream water, about pH 7.0. The pH values of the supernatant and the results of enzyme assays over the experimental period are presented in Table 5. Within 3 to 4 d of inoculation, there was significant loss of tensile strength (20%) in leaf strips with each of the four fungal species tested. Suberkropp & Klug (1981), in a pure culture study with *Tetracladium marchalianum*, found loss of strength in leaf discs of *Carya glabra* after 6 d. In the present study, the time taken for leaf strips to disintegrate through fungal activity was 9 to 12 d, compared with 10 to 12 d (Suberkropp & Klug, 1981). The differences observed may be due to differences in the chemical content of the leaves, or to differences in the enzymic capability of fungal species. It has been shown that each species of aquatic hyphomycete investigated can elaborate a characteristic array of pectinases which differ from one another in their properties.

PG, PL and PE were detected in the culture filtrates of all four fungal species grown on alder leaf-strips (Table 5). Their activity would contribute to the observed maceration and disintegration of the leaf strips, through the breakdown of pectic polysaccharides in the middle lamella and primary cell walls of the leaf lamina. Enzymic degradation of pectic polysaccharides would result in the separation of epidermal and mesophyll cells along the line of the middle lamella.

Fig. 8. Loss of tensile strength with time of alder leaf strips inoculated with (a) *Articulospora tetracladia* (■) or *Tricladium splendens* (●) and (b) *Mycoentrospora angulata* (□) or *Tetrachaetum elegans* (○). Values represent the average of three leaf strips expressed as a percentage of the uninoculated control strips. A typical 100% value for a control strip of 20×0.5 mm cross-section is 3.43×10^5 N m⁻².

Suberkropp & Klug (1981) calculated that over 50% of the weight of hickory leaf-discs were converted in 12 d from coarse particulate organic matter (>1 mm diam.) to fine particulate organic matter (<1 mm diam.). The fine particles, which are released cells, contain a lower percentage of cellulose and hemicellulose than the original, uncolonized leaf cells (Suberkropp & Klug, 1980), evidence that pectinases are probably not the only polysaccharidases involved in leaf degradation by aquatic hyphomycetes.

The levels of PG detected in the filtrates of cultures grown on leaf strips at pH 7 are comparable with those produced constitutively on a glucose substrate by *Tricladium splendens* (Fig. 1). Levels of PL and PE activity increase with time as the leaves decompose (Table 5), suggesting that these enzymes are more active around pH 7. This is supported by *in vitro* studies showing the very low levels of PG activity at pH 7 as measured on purified enzymes (Chamier, 1980), by the appearance of PL on pectic substrates of pH ≥ 6.5 , and by the alkaline pH optimum for PL. The constitutive PG may serve only for the initial attack on the pectic substrate at pH ≥ 6.5 , and the products formed may induce the synthesis of the PL, which then takes over cleavage of the substrate (Zucker & Hankin, 1970).

Suberkropp & Klug (1981) reported activity of a PG at pH 5 and an enzyme they designated as a polygalacturonic acid transeliminase (polygalacturonate lyase) at pH 8, in the culture filtrate of *Tetracladium marchalianum* growing on hickory leaf discs. They reported that the PGL was more active than the PG on leaf litter incubated in a stream of pH 7.2–8.0 at 2–4 °C. Pectolytic bacteria have been found in large populations on leaves incubated in a similar stream (Chamier & Dixon, 1982) and as polygalacturonate lyases are more characteristic of bacteria than fungi, the conclusion of Suberkropp & Klug (1981) that aquatic hyphomycetes are chiefly responsible for leaf softening may be unjustified. Furthermore, their reaction mixtures for enzyme studies of 1 ml culture filtrate or leaf homogenate (pH about 7), 1 ml 0.2 M-buffer (pH 5.0 or 8.0) and 1 ml 1% (w/v) polygalacturonic acid (pH not given), may not have had the final pH of the buffer.

Pectic substrates are not the only energy source available in leaf tissue and, until the full polysaccharidase complement of aquatic hyphomycetes is known, it must be assumed that pectinases represent only part of their enzymic capability. Moreover, it is not clear whether the

Table 5. *pH values and enzyme assays (cup-plate) on inoculated and control alder-leaf strips*

Values for pectinase assays are the radii of enzyme activity measured from the margin of the well. There was no activity in control plates. PL and PE activity could not be separated on pectin plates (pH 9) and values given incorporate both enzymes. Strips inoculated with *Tetrachaetum elegans*, *Mycocentrospora angulata* and *Tricladium splendens* had disintegrated by days 12, 11 and 9, respectively (control strips were intact).

Day	pH		Enzyme activity in culture filtrate [radius of zone (mm)]	
	Control	Culture	PG	PL and PE
(a) <i>Tetrachaetum elegans</i>				
1	7.1	7.1	—	—
3	6.7	6.5	—	—
6	6.7	7.1	2	6.5
8	6.45	6.8	1	4.5
10	6.7	7.2	1	6.5
(b) <i>Mycocentrospora angulata</i>				
1	7.1	7.1	—	—
3	6.5	6.4	—	—
6	6.3	6.8	2	4
8	6.5	7.0	1	9
10	6.6	7.0	1	10
(c) <i>Tricladium splendens</i>				
1	6.9	6.9	—	—
3	6.8	6.9	1	2
6	6.6	7.0	2	4
8	6.6	7.4	2	5
(d) <i>Articulospora tetracladia</i>				
1	6.9	6.9	—	—
3	6.8	6.9	—	—
6	6.6	7.1	1	1
8	6.7	7.0	1	3
10	6.8	7.2	2	9

relationship between pectolytic bacteria present on decaying leaves in streams and pectolytic fungi is synergistic or competitive.

The array of pectinases produced by the four organisms studied, together with their capacity to macerate leaf tissue, strongly supports the case for aquatic hyphomycetes as intermediaries of energy flow in streams. That some isolates cannot metabolize pectic substrates at low pH, despite constitutive production of PG, casts light on the distribution of species in acidotrophic and harmonic waters. There is evidence from an experiment conducted on *Tetrachaetum elegans* (Chamier, 1980) that its metabolism on pectic substrates at pH 7 is dependent on the Ca^{2+} concentration in the water. In streams of $\text{pH} \geq 6.5$, pectolytic micro-organisms utilize lyases whose activity is stimulated by Ca^{2+} . Eglishaw (1968) observed that plant degradation proceeded most rapidly in streams of high Ca^{2+} concentration and suggested that the Ca^{2+} concentration may affect the rate of microbial metabolism.

This study reveals that pectolytic capability alone does not determine whether a species will appear as a dominant or occasional colonizer of leaves (Chamier & Dixon, 1982). If competition determines the species composition within populations of aquatic hyphomycetes, then the full polysaccharidase complement of species is likely to be an important factor in the assertion of dominance, together with other physiological factors, which may include antagonism. Once an association is established, the relationship between the species may be synergistic, in the sense that the individual enzymic complement enables each organism to exploit a physiological niche which is complementary to those of others. Bärlocher & Kendrick (1974) found that when they

inoculated five species of aquatic hyphomycetes on to leaf discs, decomposition proceeded faster than it did for any species individually. This may be due to interactions between fungi which are mutually stimulating, or due to the fact that no individual species has as efficient an enzyme complex for cell-wall degradation as the range of enzymes expressed by the group as a whole.

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