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PII: S0144-8617(97)00030-1

*Carbohydrate Polymers* 33 (1997) 109–113

0144-8617/97/$17.00 + .00

# Differential degradation of apple cell walls *in vitro* by enzyme extracts from *Botrytis cinerea* and *Glomerella cingulata**

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(Received 30 April 1996; revised version received 3 December 1996; accepted 3 December 1996)

Growth and associated glycosidase enzyme production and activity of *Botrytis cinerea* and *Glomerella cingulata* were compared *in vitro* using apple cell walls as a carbon source. In liquid culture, the growth of the two pathogens was similar. Both fungi produced α-L-arabinosidase but β-D-galactosidase activity was higher in *B. cinerea*. *In vitro* degradation of apple cell walls by enzyme extracts from liquid cultures of *G. cingulata* was greater than for *B. cinerea*. After 24 h of incubation, the *G. cingulata* extract solubilized 69% of the total cell wall neutral sugar and 78% of the total uronic acid, while the *B. cinerea* extract solubilized only 43% of the neutral sugar and 37% of the uronic-acid-containing wall polymers. The neutral sugar composition of the residue remaining after degradation (the products left after the activity of the respective fungal enzymes) differed between the two fungi. © 1997 Elsevier Science Ltd

## INTRODUCTION

The gray mould fungus, *Botrytis cinerea* Pers:Fr. is second in importance only to *Penicillium expansum* Link as a postharvest pathogen of apples (*Malus domestica* Borkh.) (Rosenberger, 1990). Infection by *B. cinerea* may be initiated through wounds or at the stem or calyx end of the fruit. The maceration of host cells and further movement of the fungus within the host is facilitated by the production of pectolytic enzymes, particularly polygalacturonase (PG) (Hancock *et al.*, 1964). *Glomerella cingulata* can also cause postharvest losses but these are of lesser importance than those caused by *B. cinerea*.

One factor that may account for the relatively low virulence of *G. cingulata* is the presence of proteinaceous inhibitors of fungal enzymes in the host. Differences in inhibition of fungal pectolytic enzymes among host proteinaceous inhibitors of fungal enzymes are well known (Brown and Adikaram, 1982; Abu-Goukh and Labavitch, 1983; Brown, 1984). Several studies have shown that fungal pathogens produce pectolytic enzymes other than PG during the initial stages of infection (English *et al.*, 1971; Cooper and Wood, 1975; Anderson, 1978).

The objective of this research was to study the differences in enzymes and degradation products between the two fungi, which were grown on apple cell wall as a carbon source.

## MATERIALS AND METHODS

### Fungal culture

*B. cinerea* and *G. cingulata* were isolated from the decayed area of apple fruit 5 and 14 days, respectively, after inoculation and maintained on potato dextrose agar.

### Cell wall extraction

Cell walls were extracted from Golden Delicious apples that had been stored for 4 months at 0°C. The peel and

*Contribution for the Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel. No. 1393-E, 1996 series.

outer flesh of the entire fruit were removed to a depth of 2 mm with a mechanical peeler and discarded. The mechanical peeler, was then used to excise 2 mm of flesh tissue for cell wall extraction.

Following its removal from the fruit, approximately 100 g of apple tissue was homogenized in 100 ml of cold 80% ethanol in a Waring blender. The homogenate was filtered through two layers of Miracloth (Calbiochem) and the residue was washed with 50 ml of 80% ethanol. The ethanol-insoluble residue was used for cell wall extraction using a modification of the procedure of Rosenberger, 1990. Residue was suspended in 100 ml of 80% ethanol and heated in a boiling water bath for 5 min, stirred to break up aggregates, and filtered through Miracloth. The residue was rinsed with deionized water, suspended in 200 ml of 20 mM Hepes–NaOH (pH 7) and homogenized with a Polytron (Brinkmann Instruments) for 1 min. The resulting homogenate was filtered through Miracloth, and the residue was rinsed with Hepes–NaOH (pH 7). The residue was suspended in 100 ml of Hepes–NaOH (pH 7), placed inside a nitrogen pressure bomb at 2000 psi (140 kg/cm$^2$) for 10 min and slowly extruded from the bomb to cause complete cell disruption. The suspension was then sequentially extracted with 100 ml phenol/Tris, pH 7.5 (Huber, 1991), 200 ml chloroform–methanol mixture (1:1, v/v) and acetone by suspending the residue in the appropriate solvent, stirring for 60 min (phenol/Tris) or 10 min (chloroform–methanol and acetone), washing with two volumes of the appropriate solvent, and filtering through sintered glass. The resulting wall material was air dried overnight and then dried *in vacuo* over $P_2O_5$ at 40°C for at least 48 h.

### Fungal growth

Both pathogens were grown in 50 ml Erlenmeyer flasks containing 10 ml of modified Richard's solution (10 g litre$^{-1}$ $KNO_3$, 5 g litre$^{-1}$ $KH_2PO_4$, 2.5 g litre$^{-1}$ $MgSO_4$, 0.02 g litre$^{-1}$ $FeCl_3 \cdot 6H_2O$) and 1.0% (w/v) apple cell wall material as carbon source. A conidial suspension (0.5 ml) containing $10^5$ spores ml$^{-1}$ was added as inoculum. Flasks were incubated at 20°C for 6 days with continuous shaking at 125 rpm. Cultures were harvested at daily intervals beginning on the second day of growth and the mycelium was separated from the medium by centrifugation. Fungal growth was measured by analysing the insoluble protein content of the mycelium (Bradford, 1976).

### Glycosidase activity

An aliquot was removed from each flask of fungal culture, filtered through Miracloth, and desalted using Sephadex G-10. The desalted enzyme extract was then assayed for various glycosidase activities by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-α-D-glucoside, *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-α-D-galactoside, *p*-nitrophenyl-β-D-galactoside, *p*-nitrophenyl-α-L-arabinoside, *p*-nitrophenyl-β-D-xyloside, *p*-nitrophenyl-α-D-mannoside, and *p*-nitrophenyl-α-L-mannoside. All substrates were obtained from the Sigma Chemical Company, USA.

### Cell wall degrading ability of fungal extracts

A crude enzyme extract was obtained by concentrating the solution from each flask tenfold in a stirred ultrafiltration cell (Amicon) fitted with a PM-10 membrane. The resulting concentrate was then desalted using Sephadex G-10. In order to determine the relative ability of the crude fungal enzyme extracts to degrade apple cell wall material, a reaction mixture containing 50 mg apple cell wall, 1.5 ml enzyme extract and 13.5 ml 100 mM sodium acetate (pH 5) was incubated at 30°C for various periods of time. The reaction was stopped by boiling for 3 min. The suspension was then centrifuged at 15 000 *g* for 10 min and the supernatant was lyophylized. From the anhydrous soluble fraction, a 10 mg sample was dissolved in 2 ml 0.05 M NaCl and injected into a high performance liquid chromatography (HPLC) system with three SynChropak gel permeation columns (each 250×21 mm i.d.) run in series: GPC 4000, GPC 1000, and GPC 100. Samples were eluted with 0.05 M NaCl (pH 6.5) at room temperature. The flow rate was 4 ml min$^{-1}$ and the pressure 2250–2500 psi. Carbohydrate was detected using a refractometer; 2 ml fractions were collected and uronic acids were determined using the *m*-phenylphenol method (Blumenkrantz and Asboe-Hansen, 1973). The major carbohydrate-containing fractions were analysed for noncellulosic neutral sugar composition using gas chromatography mass spectroscopy (GC/MS) of their alditol acetates, as previously described (Gross, 1984). The pellet (insoluble material after incubation) was washed twice with 100 mM sodium acetate (pH 5) and analysed for uronic acid and neutral sugars as described above. Data are the average of three separate analyses.

## RESULTS

The growth of *B. cinerea* and *G. cingulata*, with apple cell walls as a carbon source was similar (data not shown). For both fungi, the mycelial insoluble protein content increased until day four and remained relatively constant thereafter. The only *p*-nitrophenyl-glycosidase activities detected were α-L-arabinosidase, β-D-galactosidase and β-D-glucosidase. α-L-Arabinosidase activity was higher in *G. cingulata* than in *B. cinerea* until day six, after which there was little

difference (Fig. 1(a)). β-Galactosidase activity was substantially higher in *B. cinerea* than in *G. cingulata* throughout the growth period (Fig. 1(b)). Both fungi contained relatively low levels of β-glucosidase activity, but this activity was higher in *B. cinerea* (Fig. 1(c)).

To compare the abilities of extracts to degrade apple cell walls *in vitro*, the supernatant from fungal cultures was concentrated, desalted and incubated with apple cell walls. Degradation by the *G. cingulata* extract was greater than by the *B. cinerea* extract (Table 1). After 24 h incubation, the *G. cingulata* extract solubilized 69% of the total neutral sugar and 78% of the total uronic acid residues from the wall material, while the *B. cinerea* extract solubilized only 43% of the neutral sugar and 37% of the uronic acid residues. The neutral sugar composition of the residue remaining after degradation of apple cell walls by the *G. cingulata* enzyme extract showed extensive hydrolysis, which involved all neutral sugar residues (Fig. 2). Over 50% of the rhamnosyl, arabinosyl, and galactosyl residues were solubilized in the first hour, suggesting solubilization of branched pectin. Incubation of apple cell walls with the *B. cinerea* extract resulted in less cell wall solubilization than with the extract from *G. cingulata* (Fig. 2). Arabinosyl, xylosyl, and galactosyl residues were the major residues solubilized after 5 h. Both fungi substantially degraded and solubilized arabinosyl-containing polymers.

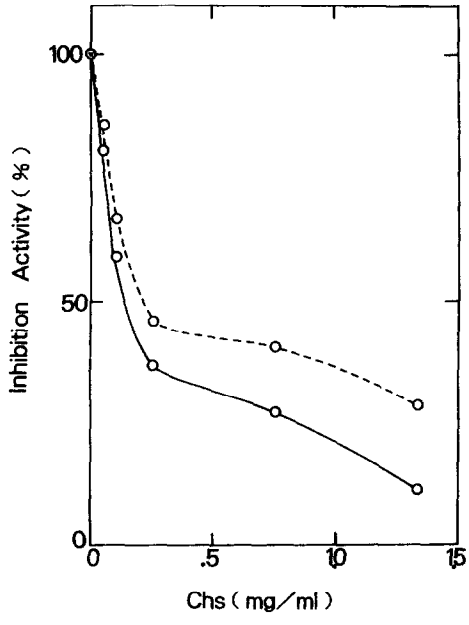


**Fig. 1.** Changes in α-arabinosidase (a), β-galactosidase (b), and glucosidase (c) during growth of *B. cinerea* and *G. cingulata*.

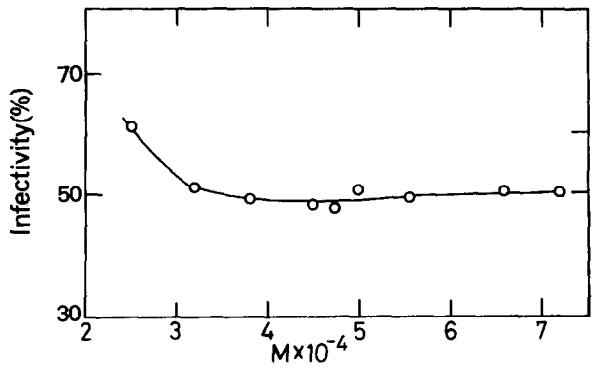


**Fig. 2.** Neutral sugar (NS) composition of insoluble material remaining after degradation of apple cell wall material by *B. cinerea* (a) and *G. cingulata* (b) enzyme extracts for various periods of time (0, 1, 5, and 24 h). Neutral sugar abbreviations: Rh, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Glc, glucose; Gal, galactose.

The relatively high (fraction A; tubes 31 to 51 DP 4-15) and low (fraction B; tubes 52 to 62 DP 14) average molecular size fractions after gel permeation HPLC results were analysed for carbohydrate content (Table 2). For both *G. cingulata* and *B. cinerea*, the neutral sugar and uronic acid contents of both fractions increased with increasing incubation time. The total amount of neutral sugar and uronic acid solubilized by the *G. cingulata* enzyme extract was higher than that solubilized by the *B. cinerea* extract.

Significant amounts of both cell wall components were released during the first hour. The amount of all neutral sugars in fraction A from incubation with *B. cinerea* enzyme extract (Fig. 3) increased during the incubation period. However, the most significant changes occurred in xylosyl, arabinosyl, galactosyl and glucosyl residues, with relatively small changes in the mannosyl and rhamnosyl contents. In *G. cingulata* (Fig. 3), fewer neutral sugar residues were solubilized, but these were released in large amounts. The amounts of arabinosyl, xylosyl and rhamnosyl residues in the

**Table 1. Total noncellulosic neutral sugar and uronic acid content of the insoluble residue remaining after incubation of fungal enzyme extracts with apple cell wall material**

| | *Botrytis cinerea* | | *Glomerella cingulata* | |
|---|---|---|---|---|
| Time (h) | Neutral sugar | Uronic acid (mg) | Neutral sugar | Uronic acid (mg) |
| 0 | 24.0±1.8 | 19.1±0.95 | 24.4±1.4 | 18.5±1.1 |
| 1 | 18.8±1.1 | 17.0±1.1 | 17.0±10.8 | 13.0±0.6 |
| 5 | 15.5±0.7 | 15.1±0.5 | 10.8±0.5 | 11.1±0.5 |
| 24 | 13.7±0.5 | 12.0±0.3 | 7.5±0.4 | 4.0±0.4 |

**Table 2. Noncellulosic neutral sugar and uronic acids content of products solubilized during incubation and separated by HPLC into relatively high (A) and low (B) average size**

| Time (h) | Neutral sugars | | | Uronic acids | | |
|---|---|---|---|---|---|---|
| | Fraction A | Fraction B | Total | Fraction A | Fraction B | Total |
| *Botrytis cinerea* | | | | | | |
| 0 | 0.20 | 0.38 | 0.58 | 0.29 | 0.11 | 0.40 |
| 1 | 1.70 | 0.52 | 0.91 | 0.45 | 0.11 | 0.56 |
| 5 | 0.56 | 1.70 | 2.26 | 0.74 | 1.19 | 1.93 |
| 24 | 1.23 | 1.88 | 3.11 | 0.95 | 2.95 | 3.90 |
| *Glomerella cingulta* | | | | | | |
| 0 | 0.20 | 0.26 | 0.46 | 0.23 | 0.00 | 0.23 |
| 1 | 1.70 | 1.40 | 3.10 | 1.50 | 0.45 | 1.95 |
| 5 | 1.60 | 3.10 | 4.70 | 1.70 | 1.50 | 3.20 |
| 24 | 3.50 | 5.20 | 8.70 | 3.00 | 4.80 | 7.80 |

The data are an average of three separate analysis.

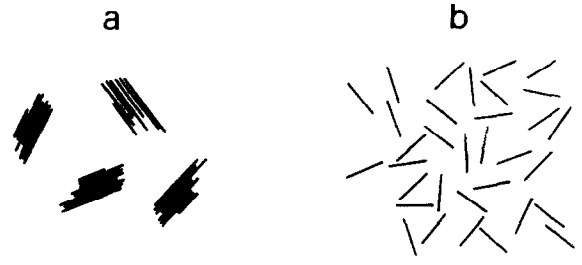


**Fig. 3.** Neutral sugar (NS) composition of HPLC fraction A product from *B. cinerea* (a) and *G. cingulata* (b) enzyme incubations. Neutral sugar abbreviations: Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Glu, glucose; Gal, galactose.

soluble product increased over time, while the galactosyl content decreased; the mannosyl and glucosyl contents remained unchanged.

In fraction B from incubation with *B. cinerea* extract (Fig. 4), the amounts of all solubilized neutral sugars increased during the incubation period. Arabinosyl residues were the predominant sugar solubilized in this fraction, followed by galactosyl, glucosyl and xylosyl residues. Fewer rhamnosyl residues were found in fraction B than in fraction A. Comparing fraction B for *G. cingulata* with fraction B for *B. cinerea* (Fig. 4) revealed that in *G. cingulata* after 5 h of incubation, only arabinosyl and galactosyl residues were solubilized, whereas xylosyl and glucosyl residues increased after 24 h and the mannosyl content remained unchanged. In fraction B for *B. cinerea*, the content of all solubilized neutral sugars increased significantly with time, reaching a maximum after 5 h incubation. Although the neutral sugar content of fraction B solubilized by *G. cingulata* enzyme extracts was much greater than that solubilized by *B. cinerea* extracts, arabinosyl comprised 45% of the neutral sugar residues solubilized by *G. cingulata* but only 33% of those solubilized by *B. cinerea*.

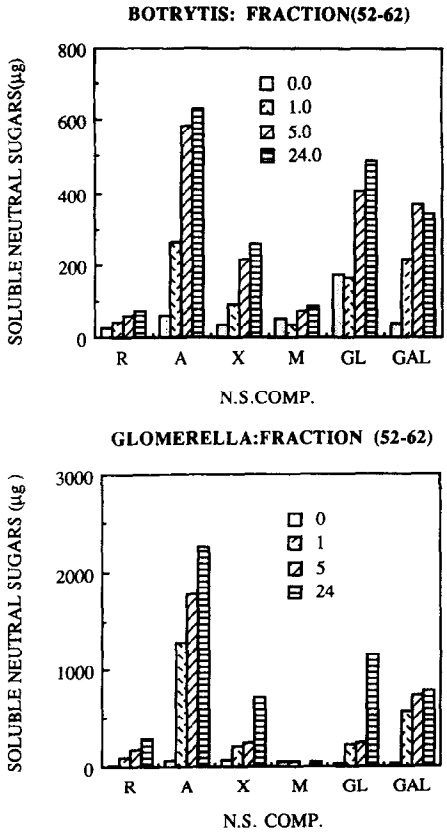


**Fig. 4.** Neutral sugar (N.S.) composition of HPLC fraction B product from *B. cinerea* (a) and *G. cingulata* (b) enzyme incubation. Neutral sugar abbreviations: Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Glc, glucose; Gal, galactose.

## DISCUSSION

The use of commercially available substrates for assaying exohydrolases (glycosidases) revealed only three active enzymes in *G. cingulata* and *B. cinerea*: α-arabinosidase, β-galactosidase and β-glucosidase. The main difference between the two fungi was in the

relatively low level of β-galactosidase in *G. cingulata* compared with that in *B. cinerea*. Since the solubilized products after incubation of apple cell walls with the enzyme extracts contained only 20–25% monomeric neutral sugars (data not shown), it was important to look for changes in endo enzymes as well. Apple cell walls provided a good, relatively natural substrate for these studies, although this material may be subject to change after treatment with 80% ethanol (Ben-Shalom, 1986).

Changes in the neutral sugar composition of cell wall during incubation with crude fungal enzyme extracts of the two fungi did not reveal any specific differences. However, the *G. cingulata* extract solubilized a greater amount of total neutral sugar than did the *B. cinerea* extract. There could have been specific differences which could be detected by glycosyl linkage methylation analysis. Such analysis might reveal differences in the enzymes between the two fungi and enable us to emphasise a specific endo hydrolase activity.

The more effective degradation of the cell wall *in vitro* but not *in vivo* by the least virulent *G. cingulata* may also be explained according to the possibility that *in situ* the pathogenic enzymes may act in synergy with the host wall hydrolyses.

In following changes in the content of neutral sugar polymers solubilized during incubation of fungal extract with apple cell walls, particularly those of relatively small average molecular size, several possible explanations may be evident. Different mechanisms may be involved in hydrolysis of the wall material *in vitro*. *B. cinerea* extract seemed to hydrolyse all neutral-sugar-containing polymers simultaneously into soluble products having relatively small molecules. However, *G. cingulata* extract primarily hydrolysed arabinosyl and galactosyl-containing polymers (branched pectin) initially and xyloglucan after further incubation.

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