Purification and characterization of four extracellular 1,3- β -glucanases of *Botrytis cinerea*

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The filamentous fungus *Botrytis cinerea* was found to produce four 1,3- β -glucanases (Glu I, II, III, IV). These enzymes were visualized by activity-staining after separation by native polyacrylamide gel electrophoresis (PAGE). During growth on glucose as the single carbon source, only Glu III was detectable in the culture supernatant of *B. cinerea*. After glucose was exhausted from the medium, the extracellular (1,3)(1,6)- β -D-glucan (cinerean) capsule of the fungus was degraded. In this phase the other three enzymes became detectable and the amount of all four enzymes increased. The enzyme with the greatest activity was Glu II, which was purified to homogeneity by SDS-PAGE. Its M_r was 75000 and its isoelectric point (pI) was 5.2. Glycosylation of Glu II was shown by the periodic acid/Schiff reaction after SDS-PAGE. Glu II cleaved cinerean and laminarin. Both substrates were degraded in an exo-manner as shown by product characterization, and by studying the viscosity decrease in comparison with the liberation of reducing groups. The concentration of substrate that gave half-maximal velocity ($S_{0.5}$) for Glu II was 580 $\mu\text{g ml}^{-1}$ for cinerean and 152 $\mu\text{g ml}^{-1}$ for laminarin. For Glu III, also purified to homogeneity by SDS-PAGE, an M_r of 84000 and a pI of 3.6 were determined. Glu III cleaved laminarin ($S_{0.5}$ 119 $\mu\text{g ml}^{-1}$) but not cinerean. Glu I and Glu IV were purified by activity-stained native PAGE. Both enzymes cleaved cinerean and laminarin in an exo-manner. Glu I focused at pI 4.9; its $S_{0.5}$ was 275 $\mu\text{g ml}^{-1}$ with cinerean and 138 $\mu\text{g ml}^{-1}$ with laminarin. The pI of Glu IV was 3.4; its $S_{0.5}$ was 171 $\mu\text{g ml}^{-1}$ for cinerean and 27 $\mu\text{g ml}^{-1}$ for laminarin.

Introduction

Many fungal 1,3- β -glucanases have been the subject of basic or applied research. Their nutritional function is evident by the extracellular degradation of 1,3- β -glucans serving as carbon sources (Bamforth, 1980; Stahmann *et al.*, 1992). Parasitic fungi may use them for degradation of the host cell wall (Jones *et al.*, 1973; Bodenmann *et al.*, 1984). Their involvement in fungal cell wall formation during hyphal tip growth, as tightly balanced antagonists of glucan synthase, is controversial (Bartnicki-Garcia, 1968, 1987; Wessels, 1988). 1,3- β -Glucanases have been studied in order to use them for various technical purposes such as preparing protoplasts (Kitamoto *et al.*, 1987), analysing fungal cell wall structure (Usui *et al.*, 1985), clarifying slimy must (Dubourdieu *et al.*, 1985),

and in the determination or degradation of barley β -glucan, which causes problems if it accumulates during the brewing process (Bamforth, 1980). In most cases, a system of 1,3- β -glucanases rather than a single enzyme was found. In the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces phaseolusporus*, seven and four different 1,3- β -glucanases respectively, have been described (Nombela *et al.*, 1988). They are distinguishable by substrate specificity, mode of action, K_m , M_r , isoelectric point (pI), localization in the culture, and conditions of formation. *Penicillium oxalicum* was found to form four enzymes during the so-called autolytic phase (Copa-Patino *et al.*, 1989). In *Penicillium italicum* (Sancez *et al.*, 1982) three developmentally-regulated 1,3- β -glucanases were identified. *Sclerotinia sclerotiorum* (Jones *et al.*, 1973) and *Sclerotium glucanicum* (Rapp, 1989) produce endo- and exo-1,3- β -glucanases. In *Trichoderma longibrachiatum* (Tangerone *et al.*, 1989) and *Trichoderma harzianum* (Kitamoto *et al.*, 1987) only a single 1,3- β -glucanase was detected.

The filamentous fungus *Botrytis cinerea* Pers.: Fr., anamorph of the ascomycete *Botryotinia fuckeliana* (Lib.) Whetzel, produces an extracellular (1,3)(1,6)- β -D-

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Abbreviations: HPTLC, high-performance thin-layer chromatography; $S_{0.5}$, substrate concentration at half-maximal velocity; TEMED, N,N,N',N' -tetramethylethylenediamine.

glucan (Montant & Thomas, 1977, 1978) termed cinerean (Dubourdieu *et al.*, 1985). This polysaccharide is produced during growth and forms a capsule adhering to the hyphae. In a recent paper (Stahmann *et al.*, 1992) it was shown that cinerean can be degraded by the fungus and can be used as the sole source of carbon and energy. This study also indicated that *B. cinerea* forms at least two 1,3- β -glucanases. The culture supernatant harvested during the cinerean formation phase degraded laminarin but not cinerean. This result is in agreement with the work of Dubourdieu & Ribereau-Gayon (1980), who described a laminarin-degrading activity which could not cleave cinerean. Urbanek & Zalewska-Sobczak (1986) studied apple-rot caused by *B. cinerea*. They found two 1,3- β -glucanases, one attacking laminarin in an exo-manner and the other in an endo-manner, but they did not examine the cleavage of cinerean.

The objective of this study was to isolate and characterize the cinerean-degrading enzyme. A further aim was to determine how many 1,3- β -glucanases are formed by *B. cinerea* and to see whether there is any influence of culture conditions or growth phase on the enzyme pattern. Are there different enzymes which can be distinguished by substrate specificity, affinity, and mode of cleavage, or is the heterogeneity a result of glycosylation or partial proteolytic digestion?

Methods

Chemicals. Laminarin (1,3- β -glucan) was purchased from Sigma. Carboxymethylcellulose was supplied by Serva. Pustulan was obtained from Calbiochem and *p*-nitrophenyl β -D-glucopyranoside from Merck. Cinerean was prepared from submerged cultures of *B. cinerea* as described previously (Stahmann *et al.*, 1992). All other reagents were analytical grade from commercial sources.

Organisms and growth conditions. *Botrytis cinerea* Pers.: Fr. strain Lu 1458 MaB (Stahmann *et al.*, 1992) and single-ascospore isolates of *Botryotinia fuckeliana* SAS 405 and SAS 56 (Faretra *et al.*, 1988) were used. The strains were cultured on solid complex medium for maintenance, and in liquid mineral salts medium for cinereanase formation, as described previously (Stahmann *et al.*, 1992).

Enzyme assays. Cinerean- or laminarin-degrading enzymes were assayed in reaction mixtures comprising 0.4 ml substrate (0.2% of the specific polysaccharide in 50 mM-sodium acetate buffer, pH 5.0) and 0.1 ml enzyme solution. The mixtures were incubated at 37 °C for 1 h and the reactions stopped by boiling the mixture briefly. Reducing sugars were determined by the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952). One unit was defined as the amount of enzyme liberating 1 μ mol of reducing equivalent (glucose was used for calibration) per min under the assay conditions. Various buffers from pH 2.0 to pH 9.0 were used to study the effect of pH on the enzyme activity: 40 mM-citrate/HCl (pH 2.0–4.0); citrate/hydrogen phosphate (pH 3.5–6.5); phosphate (pH 6.0–7.5); Tris/HCl (pH 7.5–9.0). Temperature-dependent stability was investigated by incubating the enzyme solution at different temperatures in 50 mM-sodium acetate buffer (pH 5.0) followed by a standard assay. The sugars released were analysed on high-performance thin-layer chromatography (HPTLC) Silica Gel 60 plates (Merck) as described previously (Pielken *et al.*,

1990). Additionally, sugars were separated and quantified, as their borate complexes, by anion-exchange chromatography (Carbohydrate Analyzer LC 2000; Biotronik) and detected with Cu^{2+} /bicinchoninate reagent as described by Schimz *et al.* (1985).

A second enzyme test system was based on viscometry. Four millilitres of 0.6% cinerean in 0.05 M-sodium acetate buffer (pH 5.0) was mixed with 40 μ l enzyme solution and incubated at 37 °C. To stop the reaction, the assay mixture was briefly boiled. Flow curves of this mixture containing the partially degraded polysaccharide were obtained using a Rotovisco CV 100 (sensor system Me 30; Haake). Measurements were taken at 25 °C with a linearly increasing rate of shear (0–300 s^{-1} within 8 min). The samples were presheared and, after a break of 120 s, were measured again twice to examine their reproducibility.

Each determination of enzyme activity was corrected by subtracting the absorbance or viscosity change determined in an identical assay with heat-denatured enzyme. All determinations were carried out twice, and mean values were calculated. For kinetic studies, the means and standard errors of three determinations were computed.

Isoelectric focusing. The method used was similar to that described by Robertson *et al.* (1987). Gels were poured 1.5 mm thick from the following mixture: 8.2 ml distilled water; 2 ml acrylamide mixture (30%, w/v, acrylamide; 0.8%, w/v, bisacrylamide); 0.4 ml ampholyte pH range 2.5–5.0; 0.2 ml ampholyte pH range 5–8; 1.2 ml glycerol; 19 μ l *N,N,N',N'*-tetramethylethylenediamine (TEMED); 48 μ l 10% (w/v) ammonium persulphate. The cathode solution was 25 mM-NaOH and the anode solution 20 mM-acetic acid. Samples and protein standards were mixed with an equal volume of 60% (v/v) glycerol and 4% (v/v) ampholyte of the same range used to prepare the gel. Electrophoresis was performed at 10 °C in a cooled vertical minigel system (SE 200, Hoefer) for 1.5 h at 200 V constant voltage, followed by 1.5 h at 400 V constant voltage.

Native polyacrylamide gel electrophoresis (PAGE). This technique was carried out according to Pan *et al.* (1989). Polyacrylamide resolving gels (17.5%, 1.5 mm) were prepared by mixing 9.4 ml acrylamide mixture, 3.0 ml 2 M-Tris/HCl (pH 8.8), 3.5 ml distilled water, 8 μ l TEMED and 120 μ l 10% (w/v) ammonium persulphate. The stacking gel included 0.75 ml acrylamide mixture, 1.5 ml Tris/HCl (pH 6.8), 3.6 ml distilled water, 4.4 ml TEMED and 45 μ l 10% (w/v) ammonium persulphate. A 20 mM-Tris solution, adjusted with glycine to pH 8.3, was used as the anode and cathode buffer. The gel was run for 12 h in the cooled vertical minigel system at 0 °C and 5 mA constant current.

Denaturing PAGE. Discontinuous SDS-PAGE was run in a vertical gel chamber SE 400 (Hoefer), applying the method of Laemmli (1970) using a 12.5% polyacrylamide gel.

Staining of polyacrylamide gels. 1,3- β -Glucanase staining was performed in a similar way to Pan *et al.* (1989). After electrophoresis the gel was washed three times with distilled water. Following equilibration in 0.5 M-sodium acetate (pH 4.5) it was incubated in the same buffer, containing 6.7 mg laminarin ml^{-1} , for 2 h at 37 °C. After washing the gel three times with distilled water it was heated in a boiling water bath with 1 M-NaOH, containing 1.5 g 2,3,5-triphenyltetrazolium chloride l^{-1} , until red bands appeared. The intensity of these bands was approximately proportional to the applied activity and incubation time with laminarin. The stained gels were quickly transferred to 7.5% acetic acid to reduce background staining and to conserve the contrast.

Proteins were visualized by silver staining (Meril *et al.*, 1981) to prove homogeneity in SDS and native PAGE, and by rapid Coomassie Brilliant Blue R-250 staining (Robertson *et al.*, 1987) to identify the standards in isoelectric focusing gels.

Glycoprotein visualization was based on the periodic acid/Schiff stain developed by Zacharius *et al.* (1969).

Purification procedure. The enzymes were purified from the culture broth of *B. cinerea* after cultivation for 15 d in mineral salts medium with glucose as the sole carbon source. The cell-free culture liquid was concentrated 100-fold by lyophilization and applied to a gel filtration column (Sephacryl 200 superfine, Pharmacia) equilibrated with 50 mM-Tris/HCl (pH 7.0) containing 0.5 M-NaCl. Active fractions were combined, concentrated by ultrafiltration (Ultrafree 30 kDa, Millipore) and dialysed against 5 mM-succinate/NaOH buffer (pH 4.0). This solution was applied to a carboxymethylcellulose Biogel (CM-Biogel) (Bio-Rad) column equilibrated with the same buffer. The binding glucanases were eluted by a linear gradient of 0–300 mM-NaCl after washing the column with three volumes. The glucanases which did not bind were applied to a DEAE-Sephadex A-25 (Pharmacia) column after concentration and dialysis against the column buffer (50 mM-Tris/HCl, pH 7.0). Elution was achieved by a linear gradient of 0–250 mM-NaCl. The pooled fractions of DEAE-Sephadex and carboxymethylcellulose Biogel containing glucanase activity were concentrated separately and then applied to a Sephadex G-100 column (Pharmacia), run with 200 mM-Tris/HCl pH 8.0 containing 0.1 M-NaCl. During column chromatography, protein was located by absorbance at 280 nm. The Markwell method (Markwell *et al.*, 1978) was used to estimate protein concentration of the samples, with bovine serum albumin as the standard.

Results

Conditions for 1,3- β -glucanase formation

Analysis of *B. cinerea* submerged culture supernatants by native PAGE and activity-staining with laminarin as the substrate showed that up to four 1,3- β -glucanases, termed Glu I, II, III and IV, were formed (Fig. 1).

As recently reported (Stahmann *et al.*, 1992), the formation of β -glucanases with cinerean-degrading activity (cinereanase) is regulated by the carbon source.

Fig. 1. Heterogeneity of 1,3- β -glucanase. *B. cinerea* submerged culture supernatants were analysed by native PAGE (17.5% gel); after incubation with laminarin, activity-staining was carried out. Lanes: 1, culture supernatant containing Glu I, II, III, IV; 2, purified Glu I; 3, purified Glu II; 4, purified Glu III; 5, culture supernatant containing Glu IV.

When *B. cinerea* is cultivated in mineral salts medium with glucose as the sole carbon source, cinerean is formed during the growth phase and cinereanase activity is not detectable. In contrast, laminarinase activity is present during this growth phase. When glucose is exhausted from the medium, cinereanase activity becomes measurable and both laminarinase and cinereanase activity increase.

During the growth phase on glucose, only Glu III was detectable by native PAGE. When cinerean was degraded after glucose consumption, Glu I, II and IV were additionally found. The concentration of all four enzymes increased during the course of the cinerean degradation phase. These culture conditions led to the same zymogram in *B. cinerea* Lu 1458 MaB, SAS 56 and SAS 405 cultures. Samples compared in adjacent lanes on a gel showed the same 1,3- β -glucanase pattern. When *B. cinerea* Lu 1458 MaB was cultured with cinerean or laminarin as the sole carbon source, only Glu I and Glu IV were found in the culture supernatant. When gentiobiose was used as the sole carbon source, only Glu IV was detectable. After growth with glycerol as the carbon source, no 1,3- β -glucanase formation was detectable in a culture supernatant concentrated 100-fold. It is concluded that the formation of each enzyme is regulated separately. The detection of Glu III during growth on glucose while cinereanase activity was not detectable (Stahmann *et al.*, 1992) indicated that Glu III cleaved laminarin but not cinerean.

Isoelectric points and carboxymethylcellulose-binding properties

A useful property for the distinction and separation of proteins is the pI. The 1,3- β -glucanases of *B. cinerea* focused at pH 4.9 (Glu I), pH 5.2 (Glu II), pH 3.6 (Glu III) and pH 3.4 (Glu IV) (Fig. 2). They were divided into two groups. At pH 4.0 Glu I and Glu II were positively charged while Glu III and Glu IV were negatively charged. These results were confirmed and used in a separation step with carboxymethylcellulose. While Glu I and Glu II bound to the ion exchanger, Glu III and Glu IV were eluted at about one column volume (Fig. 3).

Purification and properties of Glu II

To study the properties of the main cinereanase produced during the cinerean-degrading phase after glucose consumption, a purification procedure as described in Methods was carried out (Table 1). The cinereanase isolated in this way was homogeneous in native PAGE and was identified as Glu II (Fig. 4). The protein was also homogeneous in SDS-PAGE and had an M_r of 75000 (Fig. 5). Periodic oxidation and staining with fuchsin

Fig. 2. Determination of the isoelectric points of Glu I, Glu II, Glu III and Glu IV. The following samples were applied to a native polyacrylamide gel. Lanes: 1 and 6, amyloglucosidase (pI 3.6), trypsin-inhibitor (pI 4.6), carbonic anhydrase (pI 5.4), carbonic anhydrase (pI 6.6); 2, Glu I; 3, Glu IV; 4, Glu II; 5, Glu III. After focusing, lanes 1 and 6 were stained with Coomassie Blue; lanes 2–5 were activity-stained after incubation with laminarin. The isoelectric points of the glucanases were calculated after linear regression of R_F and pI_{standard} .

Fig. 3. Carboxymethylcellulose-binding properties of 1,3- β -glucanases. The elution profile detected at 280 nm is shown. After application of the sample the column was washed with three volumes. A NaCl-gradient was then applied. Selected fractions were analysed by native PAGE and activity-staining after incubation with laminarin.

sulphite after SDS-PAGE led to a distinct signal (Fig. 6), indicating glycosylation of the enzyme. The preparation of the sample by heating in Laemmli buffer and the following electrophoresis ensured that only covalently-bound glycosyl residues were stained (Beely, 1987). Glu II cleaved cinerean and laminarin; no activity was detectable with carboxymethylcellulose. With amylose,

Table 1. Purification of the cinerean-cleaving 1,3- β -glucanase Glu II from the culture supernatant of *B. cinerea*

Yields and purification factors were not calculated because in steps 1–4 Glu II was not the only activity determined.

Step	Total protein (mg)	Cinereanase activity	
		(U)	(U mg ⁻¹)
Culture supernatant	162	56.7	0.35
Lyophilization	104	39.5	0.38
Sephacryl-200	34	29.6	0.87
CM-Biogel	7	12.5	1.78
Sephadex G-100	2	5.4	2.72

Fig. 4. Identification of the isolated 1,3- β -glucanase as Glu II. Native PAGE (17.5%) of the purification steps. (a) Silver-staining; (b) activity-staining after incubation with laminarin. Lanes: 1, culture supernatant; 2, S-200 pool; 3, CM-Biogel pool; 4, G-100 pool.

amylopectin, glycogen and pustulan, less than 1% of the activity determined with cinerean was found. Laminari-biose and *p*-nitrophenyl β -D-glucopyranoside were not degraded. The cleavage products were identified by HPTLC as well as by anion-exchange chromatography of their borate complexes. Cinerean was cleaved to glucose and gentiobiose. Gentiobiose was not degraded. The only degradation product of laminarin was glucose. Neither the degradation of cinerean, nor that of laminarin, liberated oligomers. Furthermore, the linear decrease of viscosity and the simultaneous linear increase of reducing groups during the degradation of cinerean (Fig. 7) indicated that Glu II acted as an exo-1,3- β -glucanase (EC 3.2.1.58), cleaving one 1,3- β -linkage after the other, bypassing 1,6- β -branches. As determined by viscometry, the presence of glucose (40 mM) and gentiobiose (10 mM) during cinerean degradation had no inhibitory effect. The study of reaction rates as a function of substrate concentration fitted Michaelis–Menten kin-

Fig. 5

Fig. 6

Fig. 5. Determination of the M_r of Glu II by SDS-PAGE. The gel (12.5%) was stained with silver; 10 μ g Glu II was applied. Lanes: 1, standards [phosphorylase *b* (97300), BSA (66000), egg albumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000)]; 2, G-100 pool. M_r of Glu II was calculated after linear regression of $\log M_r$ and R_f .

Fig. 6. Staining of Glu II glycosyl moieties. SDS-polyacrylamide gel (12.5%) stained with Schiff's reagent. Lanes: 1, culture supernatant; 2, isolated Glu II.

Fig. 7. Time course of reducing group liberation and viscosity decrease. Cinerean (2 mg ml⁻¹) was digested by 1.4 nU ml⁻¹ Glu II at 37 °C and pH 5.0. After 30, 60 and 90 min the increase of reducing groups (○) was detected colorimetrically. Additionally flow curves were measured and the shear stress, τ (●) was calculated at a rate of 225 s⁻¹.

etics. A concentration of 580 μ g cinerean ml⁻¹ ($S_{0.5}$) was calculated for half-maximal velocity. For laminarin $S_{0.5}$ was 152 μ g ml⁻¹. When cinerean was used as the substrate, polymer hydrolysis was pH-dependent, showing a maximum at pH 3.5. Laminarin cleavage was less pH sensitive: at pH 6, for example, Glu II reached 85% of its maximal activity with laminarin but only 65% with cinerean. Temperature stability of the enzyme was

Table 2. Purification of the 1,3- β -glucanase Glu III

Yields and purification factors were not calculated because in steps 1-4 Glu III was not the only activity determined.

Step	Total protein (mg)	Laminarinase activity	
		(U)	(U mg ⁻¹)
Culture supernatant	162	284	1.7
Lyophilization	164	211	2.0
Sephacryl-200	31	96.1	3.1
CM-Biogel	1.5	13.6	9.1
DEAE-Sephadex	0.5	9.2	18.4
Sephadex G-100	0.3	7.1	23.7

Fig. 8. Determination of the M_r of Glu III by SDS-PAGE. The gel (12.5%) was stained with silver; 5 μ g Glu III was applied. Lanes: 1, standards [phosphorylase *b* (97300), BSA (66000), egg albumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000)]; 2, culture supernatant; 3, G-100 pool. The M_r of Glu III was calculated after linear regression of $\log M_r$ and R_f .

determined with 50 mM-sodium acetate buffer pH 5.0. Incubation at 60 °C for 10 min, and at 70 °C for 5 min, led to 99% inactivation. After incubation at 37 °C for 24 h and at 4 °C for one year, no decrease in Glu II activity was detectable.

Purification and properties of Glu III

During growth of *B. cinerea* on glucose as the carbon source, no cinereanase activity was detectable by the Nelson test, although Glu III was found in the culture supernatant by activity-stained native PAGE. This laminarinase activity was also purified to homogeneity in SDS-PAGE to compare its properties with Glu II (Table 2). The M_r was found to be 84000 (Fig. 8). No cinerean degradation was detectable by the Nelson test, vis-

cometry, HPTLC or anion-exchange chromatography. However, laminarin was hydrolysed to glucose; no laminaribiose or oligomers were detected. Therefore Glu III, like Glu II, was regarded as an exo-1,3- β -glucanase (EC 3.2.1.58), but differing in its inability to bypass 1,6- β -branches. Its affinity to laminarin ($S_{0.5}$ 119 $\mu\text{g ml}^{-1}$) was similar to that of Glu II.

Properties of Glu I and Glu IV

Urbanek & Zalewska-Sobczak (1986) described an endo-1,3- β -glucanase from the culture supernatant of *B. cinerea*. As Glu II and Glu III proved to be exo-cleaving enzymes, the remaining glucanases, Glu I and Glu IV, were studied to elucidate their specificity and mode of cleavage. Glu I was isolated by electroelution from a preparative native PAGE of culture supernatant after growing *B. cinerea* on laminarin. A preparation homogeneous in native PAGE was obtained (Fig. 1). Cinerean and laminarin were both cleaved in an exo-manner. An $S_{0.5}$ of 275 $\mu\text{g ml}^{-1}$ for cinerean and 138 $\mu\text{g ml}^{-1}$ for laminarin was calculated. Glu IV was obtained from cultures grown with gentiobiose as the single carbon source. The culture supernatant contained only Glu IV and was therefore not treated further. Glu IV had the smallest $S_{0.5}$ values of the glucanases compared in this work: 171 $\mu\text{g ml}^{-1}$ for cinerean and 27 $\mu\text{g ml}^{-1}$ for laminarin.

Endoglycosidase H and N-glycosidase F treatment

Multiple forms of *Saccharomyces cerevisiae* invertase (Trimble & Maley, 1977) and *Penicillium camembertii* lipase (Isobe & Nokihara, 1991) due to postsecretional partial deglycosylation were transformed to homogeneous deglycosylated proteins by endoglycosidase H *in vitro*. The glycosylation of Glu II has been shown above. The removal of the covalently-bound residues by endoglycosidase H and N-glycosidase F was studied. Neither had any effect on the purified enzymes or on a culture supernatant containing all four 1,3- β -glucanases. In all cases, the electrophoretic mobilities of treated and untreated glucanases in native and SDS-PAGEs were the same. The reactivity of Glu II with Schiff's reagent was also unchanged.

Protease treatment

Movahedi & Heale (1990) purified and characterized a proteinase secreted by *B. cinerea*. About 0.2 U ml^{-1} of this enzyme activity, recognizable by its pepstatin sensitivity, was found in the cinereanase-containing culture supernatant. Because Nagata *et al.* (1990a, b) found a proteolytic digestion of *Flavobacterium dormin-*

ator 1,3- β -glucanase II to 1,3- β -glucanase I, experiments with *B. cinerea* protease, proteinase K and protease type XIII were carried out. The enriched glucanases were not affected, nor did a concentrated culture supernatant containing all four enzymes change its 1,3- β -glucanases composition, as shown by native PAGE and activity-staining.

Discussion

The results of this study show that *B. cinerea* forms four different 1,3- β -glucanases. From the identical enzyme pattern of the strains Lu 1458 MaB, SAS 405 and SAS 56 we conclude a prevalent presence of this enzyme system in *B. cinerea* wild-types. All four enzymes acted in an exo-manner. The endo-1,3- β -glucanase described by Urbanek & Zalewska-Sobczak (1986) was not detected in our strains. They were working with a strain isolated from an apple, and found that the endo-enzyme was present especially on infected apple. This particular form may therefore be formed under special induction conditions or it may be a special feature of this isolate. An endo-enzyme would have been the key to a sequential induction mechanism as described for the pectic enzymes of *B. cinerea* (Leone & Van Den Heuvel, 1986). In that case, a constitutive polygalacturonase liberated galacturonic acid, which triggered a number of polygalacturonase and pectinesterase isoenzymes. The lack of an endo-1,3- β -glucanase in LU 1458 MaB, as well as the absence of oligomers during cinerean degradation *in vitro*, argues against 1,3- β -glucose oligomers as inducer molecules. As LU 1458 MaB clearly recognized cinerean (Stahmann *et al.*, 1992), the mechanism perhaps works with the undegraded macromolecule. Gentiobiose seems unlikely to function as an inducer because when used as carbon source, it had little effect on cinereanase formation, and growth on laminarin as the sole carbon source led to the same specific cinereanase activity as cinerean itself (Stahmann *et al.*, 1992).

Glu II was studied in detail because it was the dominant cinereanase during the polymer degradation phase after growth on glucose. Its M_r of 75000 is equal to that of a *Mucor hiemalis* exo-1,3- β -glucanase, and very similar to that of a *Rhizoctonia solani* enzyme (74000), both characterized by Ohno *et al.* (1986, 1989). The glycosylation of 1,3- β -glucanases as shown for Glu II is a property described for *Penicillium oxalicum* (Copa-Patino *et al.*, 1989), *Sclerotium glucanicum* (Rapp, 1989), and several yeasts (Nombela *et al.*, 1988; Villa *et al.*, 1978). Glu II cleaved cinerean and laminarin in the same way as 1,3- β -glucanases from *Trichoderma harzianum* (Dubourdieu *et al.*, 1985) and Basidiomycete strain QM 806 (Nelson *et al.*, 1969). Because of its inability to cleave gentiobiose Glu II can be used as a tool for the

Table 3. *Properties of Botrytis cinerea* 1,3- β -glucanases

Glucanase	pI	M_r	$S_{0.5}$ ($\mu\text{g ml}^{-1}$)		Mode of cleavage
			Cinerean	Laminarin	
Glu I	4.9	—	275 ± 38	138 ± 21	exo
Glu II	5.2	75000	580 ± 51	152 ± 33	exo
Glu III	3.6	84000	NAD	119 ± 22	exo
Glu IV	3.4	—	171 ± 29	27 ± 3	exo

NAD, No activity detectable.

determination of the degree of branching of 1,3- β -glucans. Interestingly, Glu III was unable to cleave cinerean. This was also found by Dubourdieu & Ribereau-Gayon (1980). They studied *B. cinerea* to find a cinereanase and found a laminarin-degrading activity unable to hydrolyse the cinerean which often appears during wine making. Copa-Patino *et al.* (1989) purified a 1,3- β -glucanase of *Penicillium oxalicum*, which also cleaved laminarin but no cinerean. They presumed an inhibition of the enzyme by melanin, which is associated with cinerean (Bull, 1970). In the case of Glu III this reason was ruled out by a direct comparison with Glu II using the same substrate charge. The ability of Glu II to digest cinerean was more sensitive to a pH change than the cleavage of laminarin. Obviously a conformational change in the enzyme caused reduced accessibility to the 1,3- β -linkages sterically hindered by the 1,6- β -glycosyl residues. An uptake of the 1,6- β -glycosyl residues into the active site could be excluded because neither glucose nor gentiobiose showed an inhibition effect. Non-competitive inhibition of *Sclerotium glaucum* 1,3- β -glucanase activity by glucose was found by Rapp (1989). The differences between Glu I and Glu IV were not as great but were still significant (Table 3). The $S_{0.5}$ of Glu IV for laminarin was one order of magnitude lower than most reported values (Schomburg & Salzmann, 1986). If the M_r values of the substrates used are assumed to be 912000 for cinerean (Dubourdieu & Ribereau-Gayon, 1981) and 5000 for laminarin (Burchard, 1985), a K_m of 0.3 μM for cinerean and of 28 μM for laminarin can be calculated for Glu IV. Only this calculation makes it clear that the enzyme has a greater affinity for cinerean than for laminarin.

The significance of *B. cinerea* 1,3- β -glucanases as catabolic tools is an obvious conclusion of our work. They are extracellular and their formation is influenced by environmental substances (Stahmann *et al.*, 1992). Three of them can be used by the fungus for the degradation of the reserve polysaccharide cinerean. The heterogeneity of these enzymes may be useful for the survival of *B. cinerea* as a relatively unspecific parasitic and saprophytic fungus. We support the isoenzyme

concept as a reason for this heterogeneity. Multiple enzyme forms could be the result of evolution from simple enzymic patterns in response to changing hosts or habit (Willems *et al.*, 1977). However, although differences in specificity and affinity are probably not due to a difference in glycosylation as shown for invertase (Lampen, 1971), and proteolytic processing was not observed as for a *Trichoderma viride* endo-1,4- β -glucanase (Stahlberg *et al.*, 1988), certainty will only be achieved by a sequence analysis of the peptides or genes.

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