Cellulolytic enzymes of the obligately biotrophic rust fungus *Uromyces viciae-fabae* are regulated differentiation-specifically

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Uredospores of the obligately biotrophic broad bean rust fungus *Uromyces viciae-fabae* form infection structures on artificial membranes providing a thigmotropic signal. In nature these are essential for invasion of the host plant through the stomata. This experimental system was used to analyse the production of cellulolytic enzymes during the differentiation of rust infection structures.

Low cellulase activity was detected in dormant spores and in germ tubes. Enzyme activity increased during appressorium development and reached a maximum when infection hyphae and haustorial mother cells were formed. Seven cellulolytic enzymes were separated by chromatofocusing on DEAE = Si500 and PBE 94 exchangers. At least two cellulases, characterized by isoelectric points of 7.1 and 7.3, were identified as *endo*-cellulases. The neutral enzyme forms increased from 3.3% of the total activity at appressorium formation to 36.6% when infection hyphae were formed (18 h p.i.), and to 45.4% in haustorial mother cells (24 h p.i.). Cellulase activity of the rust fungus is neither substrate-inducible nor catabolite repressible. The regulation of these enzymes appears to be strictly controlled by the differentiation of infection structures, and is therefore distinct from cellulases of necrotrophs and saprophytes investigated thus far.

Plant cell walls represent barriers between a host plant and an attacking pathogen. Therefore, cell wall-degrading enzymes of plant pathogenic fungi are thought to play important roles during infection (Wood, 1960; Cooper, 1984). In some interactions between plants and fungal pathogens, enzymes capable of breaching the plant's physical defence barriers are thought to be virulence or even pathogenicity factors (Köller, Allan & Kolattukudy, 1982; Crawford & Kolattukudy, 1987; Dickmann, Podila & Kolattukudy, 1989). Plant cell wall polymer-degrading enzymes of several necrotrophic fungi have, in general, been found to be regulated by substrate induction and catabolite repression (Crawford & Kolattukudy, 1987; Urbanek, 1989).

In contrast, the mechanism of cell wall penetration by obligately biotrophic fungi is barely understood. In an early publication, Van Sumere, Van Sumere-De Preter & Ledingham (1957) showed that uredospores of the wheat stem rust fungus *Puccinia graminis* f.sp. *tritici* contain cellulases and hemicellulases. In germ tubes growing on pectin agar, substrate-inducible polygalacturonase activity was found. On the other hand, Cooper (1984) reported that cellulase activity was not detectable in a number of obligate biotrophs, i.e., in uredospores of the broad bean rust and the French bean rust fungus (*U. viciae-fabae* and *U. appendiculatus*), in conidia of the powdery mildew fungus *Erysiphe graminis*, and in sporangia of

the downy mildew fungus *Bremia lactucae*. These investigations of wall-degrading enzymes of rust fungi (Van Sumere *et al.*, 1957; Cooper, 1984), do not take into account that cellulases could be formed and/or secreted at distinct stages of differentiation of infection structures by the rust fungus, i.e., when they are needed to penetrate the host cell wall. In a more recent review, Keon, Byrde & Cooper (1987) report on cellulase activity in *E. graminis* that had differentiated appressoria, and thus support the idea of differentiation-specificity of cell wall-degrading enzymes in obligate biotrophs.

Uredosporelings of most rust fungi differentiate a complex series of infection structures in order to invade the host leaf through the stomata (Mendgen *et al.*, 1988). Upon perception of the topographic stimulus provided by the stomatal guard cells (Hoch *et al.*, 1987), germ tube growth ceases and an appressorium is formed. Subsequently, the penetration peg develops, and in the substomatal chamber of the leaf the substomatal vesicle develops, which, in turn, elongates to give rise to the infection hypha. At the tip of the latter structure, the haustorial mother cell is formed upon contact with a plant mesophyll cell. Electron microscopical studies have shown that the infection peg developed by the haustorial mother cell is surrounded by a highly localized zone of disintegrated plant cell wall material (Chong, Harder & Rohringer, 1981; Taylor & Mims, 1991), suggesting the involvement of cell wall-degrading enzymes.

Since the early work of Dickinson (1949), using collodion membranes with oil inclusions to mimic the surface

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Abbreviations: CMC, carboxymethyl cellulose; h p.i., hours post-inoculation; kat, katal (mole s⁻¹); PMSF, phenylmethylsulfonyl fluoride.

topography of plant leaves, several membranes of different materials have been used to induce the differentiation of infection structures of rust fungi in the absence of the host plant (Wynn, 1976; Staples *et al.*, 1983; Hoch *et al.*, 1987). Deising, Jungblut & Mendgen (1991), using scratched polyethylene membranes, demonstrated that thigmo-induction of uredosporelings resulted in highly synchronized formation of typical infection structures of the broad bean rust fungus *Uromyces viciae-fabae*. This system enabled investigation of the formation of enzymes potentially involved in penetration of the host cell wall and their regulation during differentiation of infection structures in the absence of the host plant.

Here we use this system and show for the first time that the broad bean rust fungus *U. viciae-fabae* produces cellulose-degrading enzymes, and that these destructive enzymes are not controlled by substrate induction and catabolite repression, but are differentiation-specific. Furthermore, the substrate specificity and the extracellular/intracellular distribution of the enzymes were studied and the different enzymes were partially characterized.

MATERIALS AND METHODS

Organism

Uredospores of *U. viciae-fabae* (Pers.) Schroet., isolate I2, were used throughout this study. The uredinial culture was kept as described by Deising *et al.* (1991). Microscopical examination ensured that the uredospores produced under these conditions were not contaminated with spores of other fungi.

Differentiation of infection structures

Approximately 180 mg uredospores were dusted onto a scratched polyethylene membrane (1870 cm²). Each membrane was sprayed with 4 ml sterile distilled H₂O and incubated in the dark at 19 °C at 100% relative humidity for different periods of time. When the effect of differentiation of infection structures on enzyme formation was analyzed, thigmo-inducing polyethylene membranes and glass plates providing no thigmotropic signal (control) were used. In these experiments, uredospores that had been washed in sterile distilled water for 30 min were dried and dusted onto glass plates and polyethylene membranes.

To exclude the possibility that bacterial contaminants contribute to the cellulase activity, membranes were sprayed with 4 ml of either cycloheximide (0.1, 0.5 or 1.0 µg ml⁻¹), ampicillin (100 µg ml⁻¹), or streptomycin sulphate (100 µg ml⁻¹). All inhibitors were from Sigma, Deisenhofen, Germany.

Membranes were sprayed with 4 ml of 0.1% aqueous carboxymethyl cellulose, or with 50 mM of either glucose, fructose, or sucrose to analyze substrate induction and catabolite repression.

Preparation of crude extracts and extracellular enzymes

Crude homogenates of rust infection structures were made in extraction buffer (100 mM Na-acetate, pH 4.5, containing

1 mM PMSF, 1% (v/v) methanol, 0.1% (v/v) Triton X-100, and 200 mM NaCl). Five to ten membranes were used to measure total cellulase activity, and ten to 45 membranes were used to prepare extracts for column chromatography. Spores were homogenized by mortar and pestle; for germ tubes and infection structures a Potter-Elvehjem homogenizer (Kummer, Freiburg, Germany) was used. The homogenate was clarified by centrifugation (20 000 g, 20 min), and to the supernatant crystalline (NH₄)₂SO₄ was added to 80% saturation. After centrifugation as described above, the protein precipitate was redissolved in 10 mM (NH₄)-acetate, pH 4.9, (0.1 ml per membrane), and dialyzed (Visking dialysis tubing, 6.35 mm, exclusion size 12–14 kDa) for 16 h against 5 l of the same buffer. Remaining or newly formed precipitate was removed by centrifugation (12 000 g, 20 min) to yield the crude extract.

To obtain extracellular fungal proteins, polyethylene membranes with adhering fungal structures were inverted and floated on extraction buffer for 15 min with slow agitation (20 rpm). Thirty membranes were used to isolate extracellular material sufficient for column chromatography and subsequent measurement of enzyme activity. The washing fluid (350 ml) was concentrated to 3 ml by ultra-filtration (Amicon 8200 ultrafiltration cell and YM 5 filter, exclusion size 5 kDa) at 1.3 bar pressure. The concentrated material was dialyzed against 500 ml 10 mM (NH₄)-acetate (pH 4.9) as described above.

Sephadex G75-SF gel permeation chromatography

Samples (1.0 ml) were applied to a Sephadex-G75-SF column (378 ml gel bed, 84 cm long), equilibrated with 0.02% (w/v) NaN₃ in 0.2 M (NH₄)-acetate buffer, pH 4.9. The flow rate was 8.6 ml h⁻¹. Fractions of 2.2 ml were collected. Blue dextran (2000 kDa), BSA (67 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), and cytochrome c (12.3 kDa) (all from Sigma) were used to calibrate the column.

Chromatofocusing

In the acidic range (pH 6.0 to 2.8), chromatofocusing of crude extracts and extracellular proteins was carried out on a DEAE = Si500 (0.02–0.04 mm, Serva, Heidelberg, Germany) column (13.1 ml gel bed, 16.8 cm long). The sample was dialyzed against 25 mM histidine-HCl, pH 6.0 for 16 h. A volume of up to 3.0 ml, containing maximally 12.4 mg protein ml⁻¹, was applied to the column equilibrated with the same buffer. After adding 5 ml histidine-HCl buffer, pH 6.0, the pH gradient used to elute the proteins was generated by applying 0.5% (v/v) Servalyt 3–8 (Serva), adjusted to pH 3.9, and 0.5% (v/v) Servalyt 2–4 (Serva), pH 2.8.

Fractions containing proteins which did not bind to the DEAE = Si500 column were pooled and concentrated to 3.0 ml by ultrafiltration as described above. After adding 30 ml 25 mM Tris-acetic acid, pH 8.0, the sample was again concentrated by ultrafiltration. The concentrate (maximally 3.0 ml) was applied to a PBE 94 (Pharmacia, Freiburg, Germany) column (8.9 ml gel bed, 17.8 cm long), equilibrated with 25 mM Tris-acetic acid, pH 8.0. To elute proteins of the neutral pI range, a pH gradient was generated using Polybuffer

PB 96, (Pharmacia, Freiburg, Germany), diluted 1:13 (v/v) in distilled H₂O, adjusted to pH 6.0 with acetic acid.

The flow rates were 13.5 ml h⁻¹ (DEAE = Si500) and 25.0 ml h⁻¹ (PBE 94), the fraction sizes 1.6 and 2.1 ml, respectively.

All operations were carried out at 4°.

Enzyme assays

Cellulase. Photometric assay: To determine cellulase (*endo*- β -1,4-glucanase [EC 3.2.1.4], *exo*- β -1,4-glucanase [EC 3.2.1.91], and *exo*- β -1,4-glucosidase [EC 3.2.1.74]) activity in crude extracts and in column chromatography fractions, the assay contained 1% (w/v) carboxymethyl cellulose, Na-salt (CMC) (Serva, Heidelberg, Germany), and 0.02% NaN₃ (w/v) in 50 mM Na-acetate, pH 4.9. In a total volume of 500 μ l, 50 μ l of the crude extract or 200 μ l of the column fractions were tested. The assays were run for 2 h (crude extract) or 24 h (column fractions) at 30°. To analyse the substrate specificity of the hydrolases, microcrystalline cellulose (Avicel PH 101, particle size 38 μ m) and native cellulose (Cellulose MN 300, fiber chain length 2–20 μ m, both from Serva), 1.0% (w/v) each, were used instead of CMC as the substrate. These latter substrates were kept in suspension by shaking throughout the enzyme reaction. The formation of reducing sugars was determined photometrically at $\lambda_{500\text{ nm}}$ according to Nelson (1944) and Somogyi (1952), or at $\lambda_{410\text{ nm}}$ using *p*-hydroxy benzoic acid hydrazide as described by Lever (1972).

Viscosimetric assay: Two ml of the pooled peak-fractions of chromatofocused cellulases were added to 2 ml 1.2% (w/v) CMC and 0.04% NaN₃ in 100 mM Na-acetate buffer, pH 4.9, and time-dependent changes in velocity were taken as a measure of enzyme activity (Canevascini & Gattlen, 1981). At each viscosity determination time point, an aliquot of the reaction mixture was taken to determine the concentration of reducing sugars according to Lever (1972). Maximal available bonds were determined by reacting crude extract with the viscosity assay medium until no further alteration of the concentration of reducing sugars was detectable. The percentage of cleaved bonds was expressed on the basis of maximal available bonds.

β -D-glucosidase (EC 3.2.1.21). To 400 μ l of the chromatofocusing fractions, 100 μ l of a solution, containing 2.5 mg *p*-nitrophenyl- β -D-glucopyranoside per ml 0.4 M Na-acetate buffer (pH 5.0) was added. After incubation at 30° for 12–15 h, 1 ml 2 mM Na₂-EDTA in 1 M ammonia was added. After centrifugation (15 000 *g*, 10 min at room temperature) the absorbance of the sample at $\lambda_{405\text{ nm}}$ was determined.

Malate dehydrogenase (EC 1.1.1.37). Malate dehydrogenase activity was determined according to Sigma Technical Bulletin (No. 340-UV). The reaction mixture consisted of 136 μ M NADH and 150 μ M *cis*-oxaloacetate in 0.1 M K-phosphate buffer, pH 7.5, and 100 μ l sample in a total volume of 3.0 ml. The assay was run at 25°, and the decrease in $A_{\lambda_{340\text{ nm}}}$ was used as measure of enzyme activity.

Protein determination

Protein determination was done according to Bradford (1976),

using a commercially available dye reagent (BioRad, München, Germany), and γ -globulin as the standard.

RESULTS

Differentiation-specific formation of cellulose-degrading enzymes in *U. viciae-fabae*

The kinetics of formation of total cellulolytic enzyme activities by *U. viciae-fabae*, measured with CMC, native and micro-

Fig. 1. Time-course of formation of cellulase activity in differentiating uredosporelings of *Uromyces viciae-fabae*. Uredospores were dusted onto thigmo-inducing polyethylene membranes and allowed to form infection structures. At different time points, corresponding to different structures formed, cellulase activity was measured using CMC (●), microcrystalline (▲), or native cellulose (■) as substrate. gt = germ tube, ap = appressorium, ssv = substomatal vesicle, ih = infection hypha, hmc = haustorial mother cell. Vertical bars represent s.d.

Fig. 2. Effect of cycloheximide, ampicillin and streptomycin sulphate on the formation of cellulase activity of *Uromyces viciae-fabae*. Cycloheximide was applied at concentrations of 0.1, 0.5 and 1.0 μ g ml⁻¹. The concentrations of ampicillin (amp) and streptomycin sulphate (strept) were 100 μ g ml⁻¹. Untreated control spores (control) were sprayed with distilled H₂O. Enzyme activities of inhibitor-treated and control spores were measured after 24 h. For comparison, cellulase activity present in dormant spores (spores) was also determined.

Fig. 3. Chromatofocusing of cellulolytic enzymes of *Uromyces viciae-fabae* on DEAE = Si500 (a) and PBE 94 (b) exchangers. Crude extract from infection structures, differentiated for 24 h, was applied to the DEAE = Si500 column. Non-bound proteins were dialysed against Tris-acetate, pH 8.0, concentrated, and applied to the PBE 94 column. Proteins were eluted from both columns by applying pH gradients, and the fractions were analysed for cellulase (●) and β -glucosidase (○) activity. The dotted line indicates the pH gradient, the solid line gives the absorbance at λ 280 nm.

crystalline cellulose, is shown in Fig. 1. While low enzyme activities were found in both uredospores and germlings during growing for 4 h, activities started to increase during appressorium formation and reached a maximum at 18 h p.i., i.e. when infection hyphae developed. Similar activities were found when haustorial mother cells were produced (24 h p.i.). While the hydrolytic activity was more than ten-fold higher with CMC than with insoluble cellulose, the kinetics of enzyme formation were comparable with all substrates tested.

However, with CMC activity was still increasing at 24 h p.i., while with the insoluble substrates some decrease was observed.

When uredospores germinated on glass surfaces which provide no thigmotropic signal, only 2–5 % of the specimens differentiated appressoria and subsequent infection structures. As compared to uredosporelings differentiating on thigmotrophic polyethylene membranes where cellulase activity increased by more than 400 % (measured with CMC as the

Fig. 4. Comparison of activities of acidic (hatched bars) and neutral cellulolytic enzymes (solid bars) at different stages of infection structure differentiation by *Uromyces viciae-fabae*. Crude extracts were prepared 7, 18, and 24 h after inoculation (h p.i.). Chromatofocusing was performed as shown in Fig. 2. Cellulolytic enzymes binding to the DEAE = Si500 (acidic) and PBE 94 (neutral) column were measured as the rate of CMC hydrolysis.

Table 1. Substrate specificity of different cellulolytic enzymes of *Uromyces viciae-fabae*. Infection structures were allowed to differentiate for 24 h. Crude protein extracts were chromatofocused on DEAE = Si500 and PBE 94 exchangers, and the separated enzymes active with CMC (pI 4.5, 6.4, 7.1 and 7.3) were tested with native (Cellulose MN 300) and microcrystalline cellulose (Avicel PH 101)

Enzymes	Cellulase ^a substrates		
	CMC	Native cellulose	Microcrystalline cellulose
Crude extract	494.51 (100)	102.37 (20.70)	56.67 (11.47)
± s.d.	± 0.02	± 0.05	± 0.01
pI 4.5	39.22 (100)	7.60 (19.40)	5.59 (14.23)
± s.d.	± 4.69	± 0.63	± 0.08
pI 6.4	6.66 (100)	0.01 (0.16)	0.59 (8.92)
± s.d.	± 0.11	± 0.01	± 0.04
Mixture of pI 7.1 and pI 7.3	16.80 (100)	0.09 (0.52)	0.50 (2.95)
± s.d.	± 1.72	± 0.01	± 0.03

^a Cellulase activity in pkat ml⁻¹; the figures in brackets represent relative activity.

substrate), the enzyme activity in the controls (glass plates) increased only by 31% during 24 h.

When aqueous solutions containing 0.1, 0.5, or 1.0 µg cycloheximide ml⁻¹ were sprayed onto rust uredospores, cellulase activity measured after 24 h did not differ significantly from the activity found in dormant spores (Fig. 2). While 0.1 µg cycloheximide ml⁻¹ did not inhibit germination, only few appressoria, substomatal vesicles and infection hyphae were observed; most of these structures were abnormally shaped. At 0.5 µg ml⁻¹, cycloheximide caused reduced rates of germination, and appressoria were very rarely differentiated. At 1.0 µg ml⁻¹ only some spores were able to germinate.

Compared with the untreated control, which was allowed to differentiate infection structures for 24 h, ampicillin and streptomycin sulphate did not inhibit the formation of cellulase activity (Fig. 2) or the differentiation of infection structures.

Extracts of the broad bean rust infection structures were subjected to chromatofocusing on DEAE = Si500 (pH 6.0–2.8) and PBE 94 (pH 8.0–6.0) resins and seven enzymes exhibiting activity towards CMC were separated (Fig. 3). In the acidic range (pH 6.0–2.8), four enzymes with pIs of 6.0, 4.5, 4.0, and 3.5 were identified (Fig. 3a). In the neutral range (pH 8.0–6.0), three cellulolytic enzymes with pIs of 7.3, 7.1, and 6.4 were found (Fig. 3b). In the basic range (pH 10.5–8.5) no cellulase activity bound to or eluted from the PBE 118 exchanger. The elution profile of the cellulolytic enzymes with pIs of 6.0 to 3.5 (DEAE = Si500) precisely matched that of non-specific β-glucosidase activity, as measured with *p*-nitrophenyl-β-glucopyranoside as the substrate. In contrast, the pI 7.3 and 7.1 CMCase fractions of the PBE 94 column did not show β-glucosidase activity, whereas the peak of the enzyme with pI 6.4 only partially overlaps with a more acidic β-glucosidase peak.

Chromatofocusing was also used to visualize the developmental production of the different cellulolytic enzymes (Fig. 4). When appressoria were formed (7 h p.i.), the large majority (97%) of the cellulolytic activity bound to the DEAE = Si500 column; only 3% of the total activity was eluted from the PBE 94 exchanger in the neutral pH range. When the fungus had differentiated infection hyphae (18 h p.i.), the neutral cellulases had increased to 37% of the total cellulolytic activity. After 24 h, when approximately 15% of the fungal specimens had formed haustorial mother cells, the neutral enzymes represented 45% of the total activity.

Molecular weight and pH optima

Sephadex G-75 SF chromatography was used to determine the molecular weight of the cellulases of the broad bean rust fungus. While 17% of the enzyme activity was found in the void volume of the column, 83% eluted as a broad peak with a maximum at approximately 42 kDa and a shoulder at 48 kDa (data not shown).

The pH optimum of all enzyme forms separated by chromatofocusing was 4.9–5.0 with CMC as the substrate.

Substrate specificity of crude extract and separated cellulases, and synergism of enzymes of the cellulase complex

Crude extracts of 24 h old *U. viciae-fabae* infection structures, containing all cellulases and enzymes separated by chromatofocusing were tested for their activity on CMC and native and microcrystalline cellulose. The crude extract and all separated enzymes showed highest activity with CMC, whereas native cellulose was hydrolysed with 20.7% and microcrystalline cellulose with 11.5% of the rate found with CMC (Table 1). A similar result was obtained with the acidic cellulase (pI 4.5).

Table 2. Synergistic activities of cellulases of *Uromyces viciae-fabae* infection structures, differentiated for 24 h. Cellulolytic enzymes separated by chromatofocusing on DEAE = Si500 and PBE 94 were tested alone and in mixtures of equal volumes for their ability to hydrolyze native (Cellulose MN 300) and microcrystalline cellulose (Avicel PH 101)

Enzyme ^a	Native cellulose	Microcrystalline cellulose
pI 4.5	2532.3 ± 211.6	1862.9 ± 26.2
pI 6.4	3.5 ± 1.0	198.1 ± 12.7
pI 7.1 + 7.3	28.8 ± 0.5	165.3 ± 8.5
Σ	2564.6 ± 213.2	2226.3 ± 4.0
pI 4.5 + 6.4 + 7.1 + 7.3	4999.0 ± 217.0	3545.5 ± 332.84

^a Cellulase activity in fkat ml⁻¹.

Fig. 5. Effect of CMC, glucose, fructose, and sucrose on the cellulase activity of *Uromyces viciae-fabae*. Sterile distilled water (control), solutions containing 50 mM glucose, fructose or sucrose, or 0.1% (w/v) CMC were sprayed onto thigmo-inducing membranes inoculated with uredospores. After 24 h, infection structures were homogenized and cellulase activity was determined using CMC as the substrate. Vertical bars represent s.d.

In contrast, the enzymes characterized by pI 7.3, 7.1 and 6.4, once separated by chromatofocusing, hydrolyzed the non-soluble celluloses at very low rates.

To test synergistic effects, the separated cellulolytic enzymes were incubated with native and microcrystalline cellulose, and the activity was compared with that of the mixture of all separated enzymes (Table 2). The only enzyme showing substantial degradation of native cellulose was the acidic enzyme (pI 4.5). The neutral cellulases exhibited only traces of activity with microcrystalline and very low activities with native cellulose. Importantly, the arithmetic sum of the individual activities was only 51.3% (native cellulose) and 62.8% (microcrystalline cellulose) of the activity of all cellulases present in one reaction.

Substrate induction and catabolite repression of cellulase activity

Substrate-inducibility of cellulases and a possible repression of these enzymes by sugars was tested by spraying uredospores with either 0.1% CMC, the monosaccharides glucose and

Fig. 6. Decrease in substrate viscosity as a function of bonds cleaved by different cellulolytic enzymes of *Uromyces viciae-fabae*. Enzymes with different isoelectric points (Δ, pI 7.3; □, pI 7.1; ▲, pI 6.4; ■, pI 4.5), as separated by chromatofocusing were used in this assay. CMC served as the substrate. Reducing ends formed were measured according to Lever (1972). Maximal available bonds were determined using crude enzyme extracts containing all cellulolytic enzymes of the rust fungus.

fructose, or the disaccharide sucrose (50 mM each) immediately after inoculation of the polyethylene sheets. Addition of CMC caused no effect on the enzyme activity, but glucose, fructose and sucrose resulted in increases in cellulase activity of 43.3%, 41.2%, and 84.6%, respectively (Fig. 5).

Characterization of the mode of substrate cleavage

Four cellulolytic enzymes, separated by chromatofocusing, were compared for their ability to reduce the viscosity of their substrate CMC as a function of cleaved β-1,4-bonds (Fig. 6). Whereas the cellulases with pI 7.3 and 7.1 caused 50% viscosity reduction at 0.85% cleaved bonds, the enzymes with pI 6.4 (which showed some β-glucosidase contamination), and pI 4.5 (which co-eluted with β-glucosidase) caused 50% reduction of substrate viscosity at more than 2.2% of the maximal available bonds cleaved.

Extracellular cellulase activity

The extracellular fraction of cellulolytic activity was determined after floating the inverted membranes with adhering infection structures (24 h p.i.) on extraction buffer. For comparison, the extracellular portion of proteins and of the activity of the cytoplasmic marker enzyme malate dehydrogenase were determined as well. In contrast to protein and malate dehydrogenase activity, significant amounts of cellulase activity was found extracellularly. The large majority of extracellular cellulase activity (91.5%) was found in the acidic (pI 4.5) fraction (Table 3).

Table 3. Extracellular fraction of cellulase activity, protein, and cytoplasmic malate dehydrogenase activity in *Uromyces viciae-fabae* infection structures, differentiated for 24 h

	Extracellular fraction $\pm$ s.d.
Total cellulase activity ^a	50.6 $\pm$ 10.82
Activity of acidic cellulases ^a	46.3 $\pm$ 3.54
Activity of neutral cellulases ^a	4.3 $\pm$ 3.54
Protein ^b	7.6 $\pm$ 4.30
Malate dehydrogenase activity ^c	2.0 $\pm$ 1.39

^a Cellulase activity, ^b amount of protein, and ^c malate dehydrogenase activity are given as % of the total (intracellular plus extracellular) activity or amount.

DISCUSSION

The kinetics of formation of cellulolytic enzyme activity during infection structure differentiation of *U. viciae-fabae* has been studied in the absence of its host plant, using polyethylene membranes providing a thigmotropic signal.

Native and microcrystalline cellulose were hydrolysed at lower rates than the water-soluble model substrate CMC, but the kinetics of formation of cellulase and CMCase activity were similar. The enzymes of the cellulase complex of *U. viciae-fabae*, like those of other fungi (Fan, Gharpuray & Lee, 1987, and literature therein), appear to act synergistically in the degradation of native cellulose. Some of these enzymes, once separated from the functional unit, although clearly contributing to the cellulase complex, do not significantly degrade native cellulose but hydrolyse CMC at high rates. Therefore, although occasionally suggested otherwise (Reese, Siu & Levingson, 1950; Halliwell, 1961), CMC represents a useful substrate in biochemical studies designed to analyse kinetics of the formation and properties of the different cellulase components (Sharrock, 1988). The higher sensitivity of the assay with CMC as compared with non-soluble cellulose is of special importance since *U. viciae-fabae*, and probably the majority of obligate biotrophs, produces very low levels of cellulase activity.

Dormant uredospores contain some cellulase activity which increased during germination. Upon appressorium formation, the activity increased considerably and reached a maximum of approximately 20 times the activity found in spores when infection hyphae and haustorial mother cells were formed. The fact that the increase in cellulase activity was completely inhibited even by low concentrations of the 80 S ribosome-specific protein synthesis inhibitor cycloheximide (Obrig *et al.*, 1971), but not by high concentrations of the 70 S ribosome-specific inhibitor streptomycin sulphate (Flaks, Cox & White, 1962) or by the bacterial cell wall synthesis inhibitor ampicillin (Waxman & Strominger, 1983) indicates that the enzyme activity was not due to bacterial contaminants of uredospores. Furthermore, this result suggests that the development-related increase might be due to *de novo* synthesis of the enzymes. The latter assumption needs further experimental support, e.g. by probing blotted proteins of different developmental stages with monospecific antibodies directed against rust cellulase.

Cellulolytic activity consists of several different enzymes or (iso-) forms. Multiplicity of enzyme forms is well-documented

in fungi; multiple cellulase forms, however, can result from both, pre- and post-secretional (proteolytic) events (Niku-Paavola *et al.*, 1985; Willnick & Seligy, 1985; Messner, Gruber & Kubicek, 1988; Hagspiel, Haab & Kubicek, 1989). Analysis of *U. viciae-fabae* cellulases separated by chromatofocusing revealed that the neutral enzymes especially increased during late stages of infection structure differentiation, i.e. when infection hyphae and haustorial mother cells develop prior to penetration of the host cell wall.

Differentiation and enzyme formation by the broad bean rust fungus occur in the absence of potential inducers (cellulose) or repressors (low molecular weight sugars). The addition of CMC to infection structures did not enhance cellulase activity, but sugars such as glucose, fructose, or sucrose markedly increased the enzyme activity. This is in marked contrast to cellulases from other fungi. Cellulases have been shown to be substrate-inducible in necrotrophic fungi and in fungi non-pathogenic to plants. *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, and *Neurospora crassa* produce cellulases in culture media containing cellulose (Lumsden, 1969; Olutiola, 1978; Yazdi *et al.*, 1990; Riou, Freyssinet & Fevré, 1991). Also, catabolite repression of cellulase formation in fungi has been reported frequently. For instance, Bagga, Sandhu & Sharma (1989) demonstrated that the addition of glucose to a medium containing CMC significantly repressed all components of the cellulase complex of *Aspergillus nidulans*. Horton & Keen (1966) showed that 500 μ M glucose was sufficient to repress the cellulase of the plant pathogen *Pyrenochaeta terrestris*. The 100-fold glucose concentration (50 mM), which is in the physiological range of sugars found in plant apoplasts (Holz & Knox-Davies, 1986), did not repress cellulase activity of the rust fungus *U. viciae-fabae*. Kaminskyj & Day (1984) observed that 50 mM fructose, glucose, sucrose and trehalose induce differentiation of infection structures in the bean rust fungus *U. phaseoli* var. *typica* and result in longer growth and viability. Therefore, rust fungi are able to take up and utilize nutrients from surrounding media before having differentiated haustoria. An increase of enzyme activity in the presence of sugars could thus be due to increased viability and/or rate of differentiation of infection structures. Since rust fungi need to penetrate the host cell wall and form haustoria in the host cell, catabolite repression of cell wall-degrading enzymes such as cellulases would counteract the strategy of biotrophy, and the lack of catabolite control found for cellulases in *U. viciae-fabae* thus appears reasonable.

Chong *et al.* (1981) and Taylor & Mims (1991) have shown highly localized dissolution of the plant cell wall surrounding the infection peg of rust fungi. These and similar microscopical studies suggest the involvement of cell wall-degrading enzymes in the penetration process. In order to better understand the role played by cellulases in this biotrophic pathosystem, studies on their cleavage type and localization within the infected tissue are required. Since *endo*-cleaving enzymes are much more destructive than *exo*-acting cell wall-degrading enzymes, the cleavage type of the rust cellulases was examined. Viscosimetric measurements suggest that the cellulases with pI 7.3 and 7.1 are true *endo*-cellulases.

With uredosporelings of the broad bean rust fungus *U.*

viciae-fabae, differentiated up to haustorial mother cells, approximately 50% of the total cellulase activity was found extracellularly. This extracellular activity is mainly composed of the acidic enzyme (pI 4.5), which is able to degrade native and microcrystalline cellulose in the absence of the other hydrolytic enzymes found in crude extracts. None of the cellulases formed at late stages of infection structure differentiation, however, were detected in extracellular material. Since these *endo*-cleaving enzymes could increase the efficiency of penetration, we speculate that they are either bound to the cell wall or that their secretion is blocked until the penetration peg is beginning to form. This and subsequent structures are missing in haustorial mother cells induced on artificial membranes. Only after contact with the host mesophyll cell (Mendgen, 1982) or after induction with certain sugars (Heath, 1990), are such structures produced. However, in haustorial mother cells produced on membranes, a rim which strengthens the fungal wall around the penetration peg is observed (Freytag & Mendgen, 1991). It would be interesting to immunocytochemically determine whether *endo*-cleaving cellulases are localized in this area. Interestingly, Wolf and co-workers (personal communication, G.A. Wolf, Göttingen, Germany) have detected cellulase activity in the apoplast of French bean (*Phaseolus vulgaris*) leaves infected with the bean rust fungus *U. appendiculatus*. Taken together, these results suggest that cellulases might be involved in a coordinated manner during penetration of host cell walls by rust fungi.

This work has shown for the first time that cellulases are produced by rust fungi. The expression of cellulase activity by *U. viciae-fabae* is controlled by mechanisms active during differentiation of infection structures. Substrate induction and catabolite repression are not involved in the regulation of cellulase activity. These findings are in contrast to results obtained from other fungi, and support the concept that polymer-degrading enzymes in obligate biotrophs and necrotrophs or saprophytic fungi are regulated by essentially different mechanisms (Deising & Mendgen, 1992). Based on these data, investigations on the regulation of these enzymes at the transcriptional level are underway.

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