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Cellulase in the host–parasite system *Phaseolus vulgaris* (L.)– *Uromyces appendiculatus* [Pers.] Link

Christoph S. Schmidt[1] and Gerhard A. Wolf[2]
[1]*University of Aberdeen, Department of Plant and Soil Science, St Machar Drive, Cruickshank Building, Aberdeen AB24 3UU, Scotland, UK (Phone: 0044/(0)1224/273250; E-mail: c.s.schmidt@abdn.ac.uk);* [2]*Universität Göttingen, Institut für Pflanzenpathologie und Pflanzenschutz, Grisebachstr.6, 37077 Göttingen, Germany (Phone: 0049/(0)551/393783; Fax: 0049/(0)551/394187; E-mail: gwolf@gwdg.de)*

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Abstract

Activity of cellulase and xylanase in the intercellular washing fluid (IWF) of bean plants (*Phaseolus vulgaris*, cultivar Fori) was monitored during infection with bean rust (*Uromyces appendiculatus*). In infected plants, cellulase activity could be detected at 2 days after inoculation and reached its maximum between 7 and 8 days after inoculation. The enzyme activity was not detected in healthy controls. The cellulase had a pH optimum at pH 5.5 and a temperature optimum at 30 °C. Complete inactivation of cellulase occurred after heating to 50 °C for 30 min. In non-denaturing polyacrylamide gradient gels, the enzyme exhibited four bands (molecular masses approximately 70, 95, 120, 170 kDa). After isoelectric focusing, eight cellulase isoforms with pI values pI 4.6–4.8; 5–5.1; 5.4; 5.5; 5.9; 6; 6.5; 7 appeared. Two dimensional electrophoresis yielded 13 cellulase isoforms. Unlike cellulase, low levels of xylanase were detected in healthy controls. The activity of this hydrolase did not increase due to rust infection.

Abbreviations: AA – acrylamide; BSA – bovine serum albumin; cv. – cultivar; dpi – days after inoculation (dies post inoculationem); FW – fresh weight; IEF – isoelectric focusing; IWF – intercellular washing fluid; MES – 2-(*N*-morpholino)ethane-sulphonic acid; PAGE – polyacrylamide gel electrophoresis; pI – isoelectric point; 1D – one-dimensional; 2D – two-dimensional; SD – standard deviation.

Introduction

Plant cell walls are barriers to invading plant pathogenic fungi and bacteria. Therefore the attack of polysaccharide hydrolases (cell wall degrading enzymes) upon the host cell wall plays an important role in the infection process of those pathogens that breach the cell wall or cause maceration of the tissue (Keon et al., 1987; Cooper, 1984; Mishagi, 1982; Bateman and Basham, 1976). In some perthotrophic pathogens (*Ralstonia solanacearum*, *Pseudocercosporella herpotrichoides*), a correlation between production of polysaccharide hydrolases (cellulase, xylanase) and pathogenity could be found (Kelman and Kowling, 1965; Afshari Azad, 1992). In general, perthotrophic fungi produce large quantities of hydrolases (Cooper, 1984; Wijesundera et al., 1984) leading to breakdown of host cell walls and extensive tissue maceration.

In contrast to perthotrophic fungi, biotrophic pathogens like the rust fungi generally maintain tissue integrity and viability of their host and avoid extensive cell damage. Rust fungi penetrate their host through stomata and subsequent fungal growth (formation of the substomatal vesicle and infection hyphae) occurs in the intercellular space of the host. Upon contact of infection hyphae with mesophyll cells,

haustorial mother cells are formed (Mendgen, 1982) which penetrate the host cell wall in a highly localized manner and form haustoria (Mendgen and Deising, 1993). Ultrastructural studies showed, that in contrast to perthotrophic fungi cell wall degradation was largely restricted to these penetration sites (Bracker and Littlefield, 1973; Ingram et al., 1976; Cooper, 1981; Chong et al., 1981; Taylor and Mims, 1991) and secretion of cell wall degrading enzymes was assumed to be strictly localized (Cooper, 1984; Keon et al., 1987; Deising et al., 1995b).

Recent studies on hydrolase production by the broad bean rust *Uromyces fabae* were performed *in vitro* by cultivation of the fungus on scratched inductive membranes (Deising et al., 1991), which allowed differentiation of early fungal structures (appressoria, substomatal vesicles, infection hyphae and haustorial mother cells). A variety of cell wall degrading enzymes were detected and examined in the first 24 h of rust development: cellulases (Heiler et al., 1993), proteases (Rauscher et al., 1995), pectin methylesterases (Frittrang et al., 1992), and polygacturonate lyases (Deising et al., 1995a). The absence of substrate induction or catabolite repression of these enzymes (except for polygalacturonate lyase) and coincidence of their appearance with certain morphological stages indicated developmental regulation (Heiler et al., 1993; Deising et al., 1995b). In ungerminated uredospores, no cellulase activity was found (Cooper, 1984).

Since the fungal development on inductive membranes stops following the formation of haustorial mother cells (24 h), these *in vitro* studies were restricted to early stages prior to establishment of biotrophy. Interesting changes in the enzyme pattern, which might occur in latter stages during development in the host, or those that might be induced by the host, could not be examined. A number of rust genes have been reported to be induced *in planta* (Hahn and Mendgen, 1997). Except for chitinase and 1,3-β-glucanases (Sock et al., 1990; Fink et al., 1990), little work has been done concerning the cell wall degrading enzymes of rust fungi during development *in planta*. Here we present an examination of *endo*-acting hydrolases, particularly cellulase, appearing during growth of the bean rust fungus *Uromyces appendiculatus* [Pers.] Link in beans (*Phaseolus vulgaris* L.). As growth of the rust fungi occurs intercellularly in the apoplast, extracellular enzymes secreted into this compartment could be obtained easily in a highly concentrated and purified manner by preparation of intercellular washing fluid (IWF) according to Rohringer et al. (1983) and subjected to further characterization experiments.

Materials and methods

Culture of plants

Beans (*Phaseolus vulgaris* L.; susceptible cultivar Fori) were sown in 7×7 cm pots in a 2 : 1 mixture of compost and sand (1 plant/pot). The growth conditions in the greenhouse were 18–25 °C, 30–70% relative humidity and 16 h daylength with an illumination of 8000 lx (400 W HQL-lamps, Osram). The time-course experiment was performed in a growth cabinet under the following conditions: 20 °C, 100% relative humidity, 16 h daylength with an illumination of 5000 lx (5 Philips TL M140W/33RSv double flux fluorescent tubes).

*Inoculation with bean rust (*Uromyces appendiculatus *[Pers.] Link)*

Primary leaves of 20-day old plants were inoculated by spraying with a suspension of 2 mg/ml uredospores. Inoculated plants were subsequently incubated in a humidity chamber for 20 h (100% relative humidity, 10–20 °C, darkness). Plants identically treated, but sprayed with tap water instead of uredospore suspension, served as controls.

Preparation of intercellular washing fluid (IWF)

IWF was prepared according to the procedure described by Rohringer et al. (1983). Primary leaves were vacuum-infiltrated with double-distilled water. Infiltrated leaves were centrifuged at $1\,100 \times g$ to obtain IWF. In the time course experiment, the IWF from 3 to 4 plants was used immediately after preparation. The IWF had a pH value of 6.4–7.2.

Protein determination

Protein content was determined following the method of Bradford (1976) with BSA as a standard. The measurements were carried out in three replicates in a microplate photometer (EAR400, SLT Labinstruments, Overath, FRG).

Enzyme assay

Activity of polysaccharide hydrolases was determined with dye-conjugated substrates according to Wolf and Wirth (1990), Wirth and Wolf (1992). Activity of *endo*-acting hydrolases was detected by this method (Biely et al., 1985, 1988; McCleary 1980, 1995). The test was performed in microplates with 50 µl incubation buffer, 50 µl dye-conjugated substrate and 50 µl enzyme preparation (IWF) per well. Enzyme reaction was stopped by adding 50 µl 1 M HCl. Each sample was tested in 3–5 replicates. For optimization of test conditions, incubation temperature and buffer were varied. The following buffers were used: McIlvaine buffer (Dawson et al., 1959; stock solutions: 0.1 M citric acidic and 0.2 M Na_2HPO_4; pH range 4.3–7.6); 0.2 M sodium acetate buffer (pH range 4.5–6) and 0.2 M MES buffer (pH range 5.4–6.6). Standard incubation period was 1 h. For detection of trace amounts of cellulase in early stages of infection in the time course experiment, additional assays were run with an extended incubation time of 4 and 16 h. Enzyme activity was calculated as relative activity (in optimization experiments) or as absorbance units according to the following formula: units = $\text{absorbance}_{600\,\text{nm}} \times 1000 \times (\text{incubation time[min]})^{-1}$.

Heat treatment of IWF

To test thermal stability, cellulase activity in IWF-samples was assayed after 10 and 30 min exposure to 40, 50, 60 and 70 °C and removal of the precipitate by centrifugation (15 000 × *g*, 30 min, 4 °C).

Sample preparation for electrophoresis (poroPAGE and IEF)

The IWF used in electrophoretical separations was centrifuged at 30 000 × *g* (20 min, 4 °C), filtered through a 2 µm-Minisart disposable filter (Sartorius, Göttingen, FRG) and stored at −80 °C if not used immediately. IWF was prepared at 8 days after inoculation when cellulase activity reached maximum level in infected plants (250–450 units/ml; protein content: 0.12–0.45 mg/ml). Concentration of IWF was performed with Centricon 10 kDa-microconcentrators (Amicon Corporation, Lexington, U.S.A.) or with an ultrafiltration cell (Amicon model 8MC) using a Diaflo YM2-membrane (molecular weight cut-off 2 kDa; Amicon Corporation). Two–threefold concentration resulted in more pronounced signals, although this treatment reduced the specific activity of cellulase in IWF (8 days after inoculation) from an average 1500–2500 units/mg protein to approximately 750–1000 units/mg protein. Ten percent glycerol was added to all samples prior to electrophoresis. Results of all electrophoretic separations were confirmed by repeating the experiments with different charges of IWF prepared from plants infected with different batches of uredospores.

Non-denaturing poroPAGE

Polyacrylamide gel electrophoresis in a porosity gradient (poroPAGE) according to Margolis and Kenrick (1968), was carried out in gels with 130 mm separation distance, 1 mm thickness and a linear polyacrylamide gradient of 3–20% and 3–30% (20/1 acrylamide/bisacrylamide ratio). Instead of sucrose, glycerol was added to the acrylamide solutions (25% in the small-pore solution, 5% in the large-pore solution). The buffer system of Davis (1964) was used with slight modifications (Garfin, 1990). Non-denaturing conditions were applied to preserve the enzymatic activity of the cellulase for subsequent activity staining. Running conditions were 100 V (25 mA) for 1 h, then 200 V (30–50 mA) for 22 h, 4 °C.

Sixty microliters of IWF or 10–50 µl concentrated IWF samples were loaded per lane. For comparison, water solutions of a commercial cellulase preparation from *Trichoderma viride* (Boehringer Mannheim, FRG) were also run. BSA fraction V (Boehringer Mannheim, FRG; 20 µg per lane) served as molecular mass standard. Molecular mass was calculated according to Jeppesen (1974).

Isoelectric focusing (IEF)

Non-denaturing isoelectric focusing was performed in tube gels according to Stegemann et al. (1988). Focusing gels (length 7 cm, diameter 0.4 cm) contained 1% (v/v) servalyte pH 5–7 and 0.2% (v/v) servalyte pH 3–10 (Serva, Heidelberg, FRG) which resulted in a pH gradient from 4.5 to 7, linear in the range between pH 5 and 6.8. Cathodic solution was 0.1 M NaOH, anodic solution 0.1% H_3PO_4. Up to 200 µl IWF were loaded at the anodic side. Running conditions were 20 V/cm gel length for 1 h, then 40 V/cm gel length for

6 h, 4 °C. For pI determination, 2 marker gels loaded with Protein test mixture 9 (Serva Heidelberg, FRG; 200 µg/gel) were run additionally. Marker gels were incubated in 10% trichloroacetic acid for 20 min to remove ampholytes prior to staining with Coomassie Brilliant Blue.

Two dimensional electrophoresis

First dimension was carried out as IEF, second dimension as non-denaturing poroPAGE (70 mm × 90 mm × 1.9 mm gels) with a linear acrylamide gradient of 3–20% as described above.

Cellulase detection in electrophoretical separations

Separation gels were overlaid with a cellulase detection gel (10% acrylamide, 20/1 acrylamide/bisacrylamide, 1 mm thickness) containing 0.6% dye-conjugated substrate (carboxymethylcellulose conjugated with Remazol Brilliant Blue R; Wolf and Wirth, 1990; Wirth and Wolf, 1992). For transfer of enzyme proteins to the detection gel the capillary blot technique described by Southern (1975) was applied. Before transfer, separation gels were washed 15 min three times in sodium acetate buffer pH 5.5 (0.5 M in the first washing, then 0.1 M) to adjust their pH to cellulase optimum; detection gels were incubated in transfer buffer (0.1 M sodium acetate pH 5.5) at least 15 min. Blotting time was 17 h for one-dimensional, 23 h for two-dimensional separations followed by 1 h development of the detection gel in 0.1 M sodium acetate pH 5.5.

Results

The pH and temperature optimum of cellulase in IWF of rust-infected beans

The pH optimum curve of the cellulase in beans infected with *Uromyces appendiculatus* was narrow with a maximum at pH 5.5 in all buffers used (Figure 1). The enzyme exhibited highest activity in sodium acetate buffer. Cellulase in the IWF of beans infected with *Uromyces appendiculatus* showed maximum activity at 30 °C when incubated 1 h. However, when incubation time was prolonged up to 2 h the maximum activity level shifted to 20 °C, probably due to a degradation of the enzyme at elevated temperatures. The cellulase was heat-labile and was completely inactivated by heating of the IWF at 60 °C for 10 min or heating at 50 °C for 30 min.

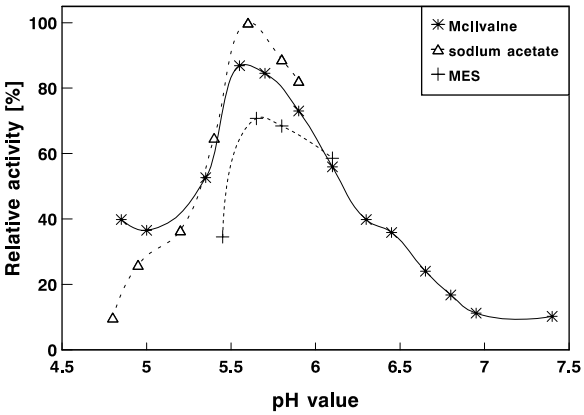

Figure 1. pH optimum curve of the cellulase in the IWF of beans (*Phaseolus vulgaris* L.; cv. Fori) infected with *Uromyces appendiculatus* [Pers.] Link measured in three different buffers (McIlvaine pH 4.3–7.4; sodium acetate pH 4.5–6; MES pH 5.4–6.6); 1 h incubation time of enzyme assay.

Time course of enzyme activity during infection

Cellulase activity could only be detected in infected plants and was completely absent in IWF of healthy control plants during all observed stages (Figure 2). In rust-infected bean plants the enzyme was clearly detectable at 5 dpi using the standard incubation time of 1 h in the enzyme assay; first traces could be measured already at 4 dpi (Figure 2). However, by extending the incubation time of the enzyme assay, cellulase activity was measurable even at earlier stages of infection: 4 h incubation allows clear detection at 4 dpi; by incubating 16 h, traces of cellulase activity could already be measured at 2 dpi. Cellulase in rust-infected plants reached a maximum level at 7–8 dpi (Figure 2) when formation of uredospores started.

In addition to cellulase, low levels of xylanase activity with a broad pH optimum ranging from pH 5–7 and a temperature optimum at 40 °C could be detected in the IWF during the whole observation period. The enzyme was also present in healthy control plants; its activity was not affected by rust infection.

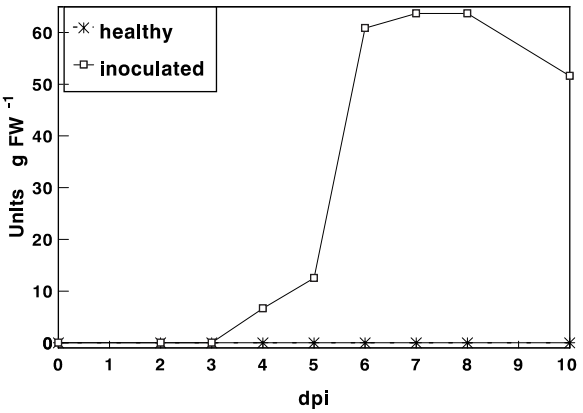

Figure 2. Time course of cellulase activity in bean (*Phaseolus vulgaris* L.; cv. Fori) during rust infection (*Uromyces appendiculatus* [Pers.] Link) compared to the uninfected healthy control. Cellulase measured in IWF prepared from 3 to 4 plants; 1h incubation time of enzyme assay; activity per gram leaf fresh weight.

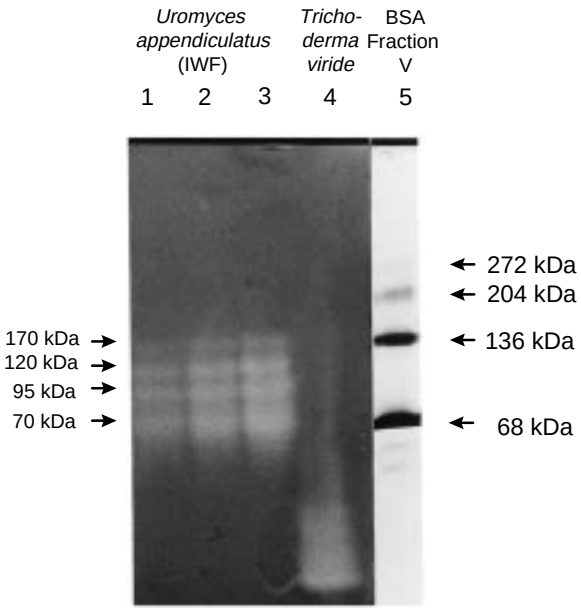

Figure 3. Zymogram of cellulase isoforms in a linear polyacrylamide gradient 3–30%: lanes 1–3: IWF from *Phaseolus vulgaris* cv. Fori infected with *Uromyces appendiculatus* [Pers.] Link 8 dpi; 10 µl, 25 µl, 50 µl IWF eightfold concentrated (13 µg, 33 µg, 65 µg protein/lane; approximately 30–90 units cellulase); lane 4 cellulase from *Trichoderma viride* (Boehringer); lanes 5: marker (BSA) coomassie-stained; molecular masses medium values from four independent experiments (polyacrylamide gradient 3–20%).

Native poroPAGE

Four bands exhibiting cellulase activity were visible after separation of IWF from *Uromyces*-infected beans (7–8 dpi) by native poroPAGE (Figure 3). Medium values (± SD) from molecular mass determinations performed in four separate experiments (polyacrylamide gradient 3–20%) were 170 ± 21 kDa, 120 ± 6 kDa, 95 ± 2 kDa and 70 ± 2 kDa (Figure 3). These molecular masses were rather high compared to the isoforms of the commercial cellulase preparation from *Trichoderma viride* (Figure 3). The largest isoform (170 kDa) was rather sensitive to longer storage of IWF in ice. As expected, IWF from healthy control plants at the same stage gave no visible cellulase bands even with high protein contents of 60 µg/lane.

Isoelectric focusing

Isoelectric focusing of cellulase isoforms in IWF of beans infected with *Uromyces appendiculatus* (8 dpi) using a pH gradient of pH 4.5–7 exhibited eight bands (illustration in Figure 4). In particular, two strong bands at the acidic end and two double bands (pI 5.4 and 5.5, pI 5.8 and 5.9; pI determinations made in one single experiment) in the middle of the gel appeared. Two faint bands represent near-neutral isoforms (pI 6.5; pI 7; Figure 4). Separation of highly concentrated IWF (300 units per lane) in a pH gradient of pH 3–10 showed no bands outside the pH range from pH 4.5–7.

Two-dimensional separation of cellulase from beans infected with Uromyces appendiculatus

Thirteen isoforms of cellulase (8 dpi) were visible after two-dimensional separation of IWF (Figure 5). Spots were numbered as illustrated in Figure 4. Isoform pattern and pI determination were reproduced in 8 experiments. No molecular mass determinations were made but spots were aligned to the cellulase bands appearing in IEF and poroPAGE according to their horizontal and vertical position (Figure 4).

An accumulation of isoforms in the more acidic range (pI 4.5–5) was observed (Figures 4 and 5). In the second dimension, the two first IEF-bands (pI 4.6–4.7 and pI 5.0–5.1) divided up into several isoforms with different molecular masses (isoforms No. 1–4 and isoforms No. 5–7 respectively; Figure 4) which could be aligned to the 70 kDa-, 95 kDa- and 120 kDa-band, respectively. Slight differences in pI and molecular mass of these isoforms (No. 1–7) were only visible

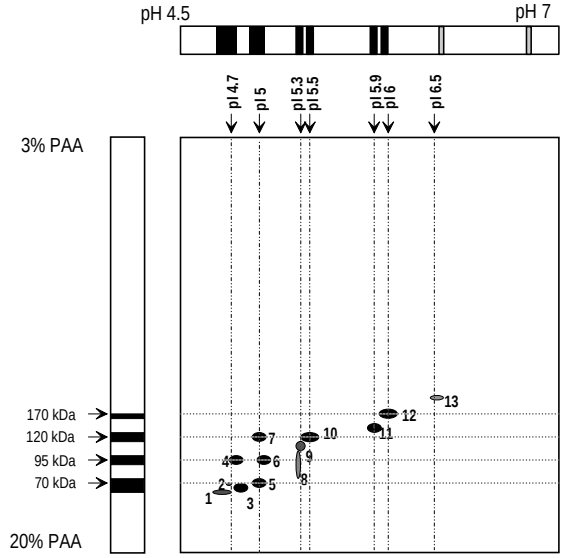

Figure 4. Illustration of the two-dimensional pattern of cellulase isoforms in IWF of *Phaseolus vulgaris* cv. Fori infected with *Uromyces appendiculatus* [Pers.] Link; and corresponding one-dimensional patterns (first dimension isoelectric focusing, second dimension poroPAGE) isoforms numbered; pI values are the average of values of 8 independent experiments; molecular masses: no measurement in 2D-electrophoresis, values assumed according to alignment of the spots to 1D-poroPAGE-bands.

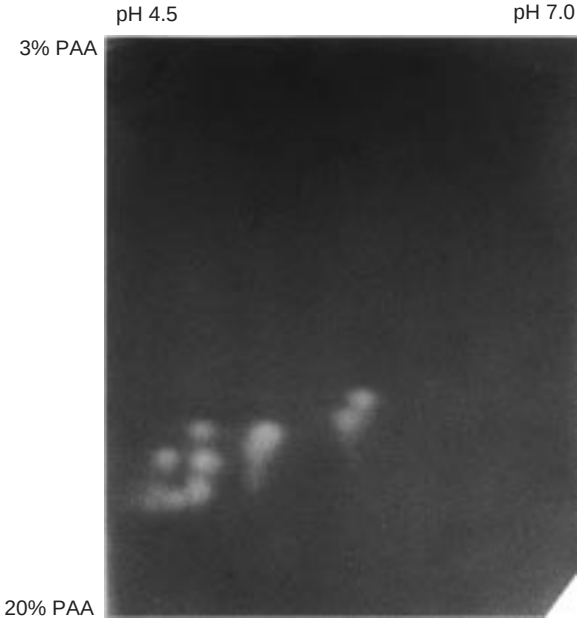

Figure 5. Two-dimensional pattern of cellulase isoforms in IWF of *Phaseolus vulgaris* cv. Fori infected with *Uromyces appendiculatus* [Pers.] Link 8 dpi; first dimension IEF, pH gradient pH 4.5–7, second dimension poroPAGE, polyacrylamide gradient 3–20%; 200 µl threefold concentrated IWF (120 µg protein, 90 units cellulase).

in two-dimensional separations (Figure 4). Isoforms No. 4 (pI 4.6–4.7) and No. 6 (pI 5–5.1) had exactly the same molecular mass. They form presumably the 95 kDa band in one-dimensional poroPAGE.

The isoforms with medium pI (pI 5.3, 5.5, 5.9, 6) appearing in the IEF double bands showed a similar pattern (Figures 4 and 5): the slightly more acidic neighbour (No. 9 and 11) had a slight lower molecular mass than its less acidic neighbour (No. 10 and 12). No. 10 could be aligned to the 120 kDa band, No. 12 to the 170 kDa band. Their acidic neighbours could not be aligned to any poroPAGE band (Figure 4). Presumably, they are present in the background visible around the poroPAGE bands (Figure 3). Below isoform 9, an additional faint spot was visible (No. 8, Figures 4 and 5).

The pI 6.5 isoform (No. 13) was only faint in two-dimensional separations and not visible in all 8 experiments. The neutral isoform (pI 7) was not detectable in 2D separations (Figure 5).

Discussion

Cellulase activity was only present in bean plants infected with *Uromyces appendiculatus* and clearly absent in healthy control plants. This gives rise to the assumption that the enzymes are of fungal origin but the possibility that they are plant enzymes induced by the fungus cannot be excluded with absolute certainty. Acidic cellulases with similar pI values (pI 4.5 and pI 4.8) and pH optimum (pH 5–6) as observed here were reported in *Phaseolus vulgaris*; they were auxin-regulated (Durbin and Lewis, 1988). An induction by the fungus seems possible since rust fungi can increase auxin levels in their hosts (Shaw and Hawkins, 1958; Daly and Inman, 1958; Pilet, 1953; Hirata, 1954). An increase of cytokinin content was reported for *Uromyces*-infected *Phaseolus*-beans (Kiraly et al., 1966, 1967). Also, an alteration of ionic strength induced by the intercellularly growing rust hyphae may lead to the release of tightly wall-bound

plant cellulases into the apoplastic fluid (R. Rohringer, personal communication).

However, in other (perthotrophic) pathogens of the bean plant (*Xanthomonas campestris* pv. *phaseoli*), cellulase activity *in planta* was correlated with cellulase activity *in vitro* and with the amount of pathogen present in the plant (Goodwin et al., 1995), which indicates a microbial origin of the enzyme rather than an induction of cellulases through the pathogens in the bean plant. In addition, as cellulase activity has been demonstrated in other rust species (Van Sumere et al., 1957; Heiler et al., 1993) a fungal origin of cellulase isoforms in rust-infected beans seems highly probable.

Earlier studies on the plant cell wall degrading enzymes of rust fungi, particularly the closely related *Uromyces fabae* (Heiler et al., 1993; Rauscher et al., 1995; Frittrang et al., 1992; Deising et al., 1995a) focused on 24 h development *in vitro*. Heiler et al. (1993) showed a strictly differentiation-dependent regulation of cellulase activity in *Uromyces fabae*. *Endo*-cleaving neutral cellulases arose later in the development than the acid isoforms and contributed a minor portion of total activity but their relative amount rose during development when haustoria mother cells are formed (37–45% in 18–24 h old rust germlings). The neutral *endo*-cellulases were not secreted at this stage. In our investigation, no *endo*-cellulase activity was detectable in IWF at comparable early infection stages (0–2 dpi). Also, no increased xylanase and protease activity could be measured in the IWF of infected plants during the whole observation period, although extracellular *endo*-proteases have been reported in the closely related *Uromyces fabae* (Rauscher et al., 1995; Clement et al., 1993). Apparently, their activity was too low to be detected in the intracellular washing fluid despite the high inoculum applied here. These data support the hypothesis of a highly localized cell wall degradation and a very limited secretion of *endo*-acting cell wall degrading enzymes by the biotrophic rust fungi at the initial stage of infection (Cooper, 1984; Keon et al., 1987; Deising et al., 1995b). This would avoid host tissue breakdown and limit elicitation of host defense responses (Deising et al., 1995b). At least, *endo*-xylanase was reported to be a potent elicitor in tobacco (Lotan and Fluhr, 1990).

However, contradictory to the hypothesis of a cautious and sparse secretion of cell wall degrading enzymes by rust fungi, *endo*-cellulase activity in the apoplast rose to high levels in the later stages of bean rust infection (6–10 dpi). The assumption seems reasonable that cellulases serve for different functions in early and in latter stages of fungal development. Cell wall degrading enzymes function in the early stages of infection to facilitate localized penetration of the cell wall for haustoria formation. A sparse local secretion is sufficient for this purpose (Deising et al., 1995b) and thus no enzyme activity is detectable in the IWF during the first days of rust infection. Contrary to this, the main function of the cellulases in later infection stages may be the utilization of the host cell wall as additional carbon source rather than the localized penetration of the host cell wall as a barrier. Cellulase peak level (7–8 dpi) coincided with formation of uredospores. At 8 dpi, senescence of the leaves due to exhaustive stress was visible. Content of soluble sugars (glucose, fructose) in the apoplast of beans infected with *Uromyces appendiculatus* decreased rapidly when formation of uredospores began (Wagner and Boyle, 1995), and as nutrient demand reached peak level for production of spores; therefore, an opening up of additional carbon sources may be essential. Rust fungi are able to take up nutrients like glucose and trehalose, the degradation products of cellulolytic decay, not only via haustoria but also via the plasmalemma of their hyphae (Kaminskyj and Day, 1984). In this context it is noteworthy that in *Uromyces fabae* a membrane bound H^+-ATPase, which possibly serves for glucose uptake, is localized in haustoria, and to a lesser extent also in germtube plasmalemma (Struck et al., 1995). Thus, nutrient uptake seems likely to take place not only by haustoria but also in the apoplast by intracellularly growing hyphae and could use the degradation products released into the intercellular space. High cellulase activities, not restricted to the haustorial sites may be advantageous for this nutritional purpose. A great number of perthotrophic pathogenic fungi can grow on cell wall material of their hosts as sole carbon source (Bateman et al., 1969; Anderson, 1978; Cooper et al., 1988), which further demonstrates the usefulness of extracellular hydrolases not only for cell wall penetration, but also for nutrition of the pathogenic fungi.

The observed pH optimum of the cellulase in the IWF of beans infected with *Uromyces appendiculatus* is very common among fungal enzymes although the optimum curve is remarkably narrow. Cellulases of *Puccinia graminis* and *Uromyces fabae* have similar pH optima (Van Sumere et al., 1957, Heiler et al., 1993; Table 1). In this context it is important to note that the measured pH (6.4–7.2) of the IWF lies much above the pH optimum of the cellulase in IWF. A lower

Table 1. Comparison between physicochemical properties of the cellulases detected in *Uromyces fabae* after 24 h development *in vitro* (Heiler et al., 1993) and cellulases present in the IWF of bean (*Phaseolus vulgaris* L.) after 7 day development of *Uromyces appendiculatus* [Pers.] Link

	Uromyces fabae, 24 h (1 day) Development *in vitro* (Heiler et al., 1993)	*Uromyces appendiculatus*, 7 days, Development *in planta* (this study)
pI (Cleavage type, localisation)	3.5 4.0 4.5 (*exo?*, extracellular) 6.0 6.4 (*exo*?) 7.1 (*endo*) 7.3 (*endo*)	4.6–4.8 (*endo*[a], extracellular) 5.0–5.1 (*endo*, extracellular) 5.3 (*endo*, extracellular) 5.5 (*endo*, extracellular) 5.9 (*endo*, extracellular) 6 (*endo*, extracellular) 6.5 (*endo*, extracellular) 7 (*endo*, extracellular)
Molecular mass (kDa)	42, 48 (83% of activity); > 67 (17% of activity) (determined by gel filtration)	70, 95, 120, 170 (determined by native poroPAGE)
pH Optimum	4.9	5.5

[a]The applied cellulase assay detects *endo*-acting activity (Biely et al., 1985, 1988; McCleary, 1980, 1995) but the association of *exo*-acting compounds with the detected *endo*-acting activities in a cellulase complex cannot be excluded.

pH value in the host cell wall than in the IWF may exist due to the carboxylic groups of uronic acid components in the pectins and hemicellulases. Cellulases of *Phaseolus vulgaris* also have their optimum in the range of pH 5–6 (Durbin and Lewis, 1988). A pH value of 4.5–5 is reported for the apoplast of broad bean (*Vicia faba*; Aloni et al., 1988). Under such acidic conditions, the near-neutral isoforms (pI 6.5, pI 7) would be highly positively charged and thus bound to the negatively charged plant cell wall (see also Deising et al., 1995b). This may be a cause for their low activity of in IWF.

Slight pH changes (0.5–0.8 pH units) were reported in the IWF of rust-infected plants (Deising et al., 1995a; Tetlow and Farrar, 1993). On the contrary, no pH changes were observable in the IWF of *Uromyces appendiculatus* infected french beans compared to the control plants. Highly localized pH alterations at the haustorial penetration sites as assumed by Deising et al. (1995b) may remain undetected in IWF and thus nonexclusive.

The cellulase isoforms present in beans infected with *Uromyces appendiculatus* at 8 dpi have considerable higher molecular weights than the cellulase isoforms in *Uromyces fabae* at 2 dpi (Table 1). In *Uromyces fabae* cellulases of low molecular mass (42 and 48 kDa) predominate and isoforms of higher molecular weight contribute to only 17% of total cellulase activity at early developmental stages (Heiler et al., 1993; Table 1). The fraction of higher molecular weight might presumably rise during later stages of development and might contain isoforms homologous to the cellulases described here as some *Uromyces fabae* isoforms exhibit similar pI's (pI 4.5, pI 6.0, pI 6.4, pI 7; Heiler et al., 1993; Table 1). Also, low molecular mass isoforms may form multimers of high molecular mass as reported for cellulases in other fungi (Goyal et al., 1991). Another possibility may be the induction of new fungal cellulase genes by the host in the later stages of fungal development *in planta*. Thirty-one rust genes were reported to be *in planta*-induced (Hahn and Mendgen, 1997). However, with the data obtained so far all assumptions about the origin of single cellulase isoforms in *Uromyces* remain speculative.

To elucidate the question on the definite origin of the cellulase isoforms it is necessary to construct a cDNA-library of rust-infected beans in expression vectors and to isolate cellulase clones using activity screening according to Wolf and Wirth (1990) for further characterization. However, posttranslational mechanisms like variable glycosylation (Gum and Brown, 1977; Willick and Seligy, 1985) and proteolytic processing

and digestion (Hagspiel et al., 1989; Kubicek-Pranz et al., 1991; Mo and Hayashida, 1988; Van Tillbeurgh et al., 1986) are common causes for multiplicity of fungal cellulase isoforms and these processes may be altered in vector organisms (Hazlewood et al., 1992; Pentilla et al., 1987). Thus, the examination of isoforms in their natural environment, as done here, is of crucial importance for comparison.

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