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Chitinases and β-1,3-Glucanases in the Apoplastic Compartment of Oat Leaves (*Avena sativa* L.)[1]

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ABSTRACT

To isolate chitinases and β-1,3-glucanases from the intercellular space of oats (*Avena sativa* L.), primary leaves were infiltrated with buffer and subjected to gentle centrifugation to obtain intercellular washing fluid (IWF). Approximately 5% of the chitinase and 10% of the β-1,3-glucanase activity of the whole leaf were released. Only small amounts (0.01–0.03%) of the intracellular marker malate-dehydrogenase were released into the IWF during infiltration. Activities of chitinase and β-1,3-glucanase in the IWF and in the leaf extract were compared by different chromatographic methods. On Sephadex G-75, chitinase appeared as a single peak (M_r 29.8 kD) both in IWF and homogenate. β-1,3-Glucanase, however, showed two peaks in the IWF (M_r 52 and 31.3 kD), whereas the elution pattern of the homogenate showed only one major peak at 22 kD. Chromatofocusing indicated that the IWF contained four chitinases and five β-1,3-glucanases. The elution pattern of the homogenate and IWF were similar with regard to the elution pH, but the peak intensities were distinctly different. Our results demonstrate that extracellular β-1,3-glucanases are different from those located intracellularly. Extracellular and intracellular chitinases do not differ in molecular properties, except for one isozyme which seems to be confined to the extracellular space. We suggest that both enzymes might play a special role in pathogenesis during fungal infection.

Many plants possess chitinases (EC 3.2.1.14) and β-1,3-glucanases (EC 3.2.1.6), but their function in plant metabolism is not clear (2). Many plant pathogenic fungi contain chitin and β-1,3-glucans as cell wall components. Therefore, numerous authors propose that these enzymes might act as a defense mechanism against fungal pathogens (3). Most of the previous *in vitro* studies on the ability of these enzymes to attack fungal cell walls did not take into consideration their localization in the plant cell. Many hydrolytic enzymes thought to be involved in defense reactions are almost exclusively located in the vacuole (1). Also, after induction of chitinase in bean leaves by ethylene treatment (5), this enzyme was found mainly in the vacuole but not in the cell wall. In this case, the enzymes would intervene in pathogenesis only very late, *e.g.* in a hypersensitive (5) or a necrotrophic reaction.

Many biotrophic parasites grow mainly in the intercellular space where the plant cell wall forms the contact surface between fungus and plant. Even if they differentiate haustoria within the cell, the plasma membrane is not penetrated. Infection structures of rust fungi have chitin and β-1,3-glucan in their cell wall, but their wall components are differently exposed during differentiation of these infection structures (13). Germ tubes grow on the leaf surface and have chitin on their exterior wall layer (13). After penetrating through the stoma into the intercellular space, the differentiating infection hypha has an inner layer containing chitin (7) that is covered with a nearly chitin-free outer layer (7, 13) consisting mainly of glucans (13). This outer layer may contain race and species specific carbohydrates (13, 14). Because of these specific surface components, highly specific plant hydrolases could be involved in recognition or defense mechanisms. We investigated whether there are extracellular chitinases and β-1,3-glucanases which are already present in the uninfected plant and which might contact the fungus at an early infection stage. To study the function of these extracellular chitinases and β-1,3-glucanases, they must be isolated from the intercellular space in order to distinguish them from intracellular enzymes. In this work, we present the results from noninfected plants to show the normal enzyme profile in the absence of fungus.

MATERIALS AND METHODS

Chemicals. Colloidal chitin was prepared according to Lingappa and Lockwood (12). ^{3}H-labeled regenerated chitin was synthesized according to Molano *et al.* (16). Lobster chitin, bovine serum albumin, ovalbumin, chymotrypsinogen A, horse myoglobin, and Servalyt were obtained from Serva, Heidelberg, F.R.G.

Laminarin from *Laminaria digitata*, azocoll, and chitosan were purchased from Sigma. ^{3}H-labeled acetic anhydride was from Amersham, U.K. Scintillation cocktail Ready-Solv HP was obtained from Beckman. All chemicals used for reagent and buffer preparations were of analytical grade.

Plant Material. Seedlings of *Avena sativa* L. cv Selma were grown in compost soil in the greenhouse. Summer conditions were: natural light period with 7,500 to 11,000 lux, 20°C to 25°C. In winter, plants were additionally illuminated with Osram HQI-E 400 watt bulbs for 6.5 h daily. This produced a total light intensity of 3500 lux approximately 10 cm above the plants. The temperature was 18°C. Primary leaves of 10 d old plants were harvested.

Infiltration of the Leaves and Preparation of IWF.[2] Infiltration was carried out according to Rohringer *et al.* (21) with the following modifications: the cut ends of the leaves were rinsed three times in deionized water prior to infiltration. Five hundred mL of a 100 mM sodium phosphate buffer (pH 6) was used to infiltrate 12 g of approximately 6 cm long leaves. The leaves were infiltrated in a desiccator for 15 min using a water pump. To obtain the IWF, leaves were centrifuged at 1200 rpm in a

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[2] Abbreviations: IWF, intercellular washing fluid; MDH, malate dehydrogenase.

Sigma 2KD (Sigma) centrifuge with their tips pointing to the bottom of the tube.

The IWF was frozen at −20°C immediately after centrifugation and could be stored in this state for at least one year without any notable loss of chitinase and β-1,3-glucanase activity. Prior to further use, the sample was thawed at 38°C and then centrifuged (20,000 rpm, 30 min, Sorvall SS34 rotor).

Multiple Infiltration. For multiple infiltration, the leaves were infiltrated and centrifuged up to six times. For comparison, homogenates were prepared from noninfiltrated and infiltrated leaves as described below.

Preparation of the Homogenate. The leaves were homogenized in 100 mM sodium phosphate buffer (pH 6, 6 mL/g) with sea sand using a mortar and pestle. The homogenate was then filtered through four layers of gauze bandage, and the filtrate was centrifuged for 30 min at 20,000 rpm (SS34 rotor) and stored at −20°C.

Chitinase and β-1,3-glucanase activity of IWF and homogenate were compared on the basis of leaf fresh weight.

Assay for β-1,3-Glucanase. The enzyme assay contained between 20 and 100 μL enzyme solution, 250 μL laminarin (2 mg/mL laminarin in 50 mM potassium acetate buffer, pH 5), made up to 500 μL with 50 mM potassium acetate buffer, pH 5. The assays were carried out at 38°C for 1 to 4 h, in a few cases up to 15 h. Then 500 μL of copper reagent (25) was added to the solution, which was then boiled in a water bath for 10 min and quickly cooled down to room temperature. After adding 1 mL of arsenomolybdate color reagent (18), the optical density was measured at 500 nm against a buffer blank.

Assay for Chitinase. (a) *Colorimetric assay.* The colorimetric assay was carried out using a modification of Boller *et al.* (4). The assay mixture contained the enzyme solution, 200 μL of 50 mM sodium acetate buffer, pH 4.5, and 1.5 mg of colloidal chitin in a total volume of 400 μL. It was incubated at 38°C for 16 to 20 h and then centrifuged (12,000*g* for 5 min). To 300 μL of the supernatant was added 100 μL of 0.2 M potassium tetraborate, and the amount of liberated *N*-acetyl-glucosamine (NAcGlc) was determined according to Reissig *et al.* (20). The controls included enzyme and substrate blanks as well as internal standards. Activity was determined from a calibration curve according to Boller *et al.* (4). (b) *Radiometric assay.* Radioactive ^{3}H-labeled chitin was used as the substrate (Molano *et al.* [16]). The reaction mixture contained the enzyme solution, 120 μL of 50 mM sodium acetate buffer, pH 4.5, and tritium-labeled chitin (5 kBq) in a total volume of 250 μL. The solution was incubated at 38°C for 2 h, after which the reaction was stopped with 250 μL of TCA. After centrifugation (12,000*g* for 5 min), 100 μL of the supernatant was carefully removed and its radioactivity determined.

Other Enzyme Assays. NAD MDH (EC 1.1.1.37) activity was determined according to the Sigma Technical Bulletin (23). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was determined according to Tschen (26), and the activity of catalase (EC 1.11.1.6) according to the Sigma catalog (22). Proteolytic activity was measured according to Ragster and Chrispeels (19) using the unspecific substrate azocoll. Tests were performed at the pH of the extraction buffers (pH 6).

Protein Determination. Protein content was determined according to Bradford (6) using the Bio-Rad protein assay kit. Protein in column eluates was monitored by measurement of the absorption at 280 nm.

Sample Preparation and Column Chromatography. All procedures were carried out at 10°C. Prior to column chromatography, the samples were concentrated by ultrafiltration using an Amicon 8200 apparatus with a YM5 filter (exclusion size 5000 D). If necessary, water was added to reduce the ion strength. Except for separation on Sephadex G75, starting buffer was added to the concentrated solution to obtain the desired pH. The chromatography columns were poured according to manufacturer's instruction.

HPLC. HPLC was carried out using an LKB single-pump gradient system with an LKB 2151 Variable Wavelength Monitor. The effluent was monitored at 280 nm.

TLC. Reaction products released from laminarin by the different β-1,3-glucanases were chromatographed on Silica Gel 60 plates (Merck) in the solvent system 2-propanol:water:ethyl acetate (7:2:1, v/v) according to Hien and Fleet (8). Compounds were detected by spraying the plates with 0.5% (w/v) thymol in ethanol, containing 5% (v/v) sulfuric acid, and then heating for 15 min at 110°C.

SDS-PAGE. Slab gel electrophoresis was carried out according to Laemmli (11) using a 11% separation gel. Silver nitrate stain was used according to Morrissey (17); step two was omitted.

Molecular Weight Determination. Molecular weight was determined by gel chromatography on Sephadex G-75 using Serva protein standards and dextran blue as void volume marker. For SDS-PAGE, Bio-Rad SDS-PAGE Molecular Weight Standards Low were used.

RESULTS

Isolation of Intercellular Proteins. Single infiltration of oat leaves with buffer released approximately 2 to 4% of the whole leaf chitinase and β-1,3-glucanase activity. The protein content of the IWF was 0.044 mg per gram fresh weight. Different preparations varied by 7% in protein content. MDH activity, which was used as a marker for intracellular proteins in the IWF, accounted for 0.01% to 0.03% of total extractable activity. Other intracellular marker enzymes, catalase and glucose-6-phosphate dehydrogenase, were not detected in the IWF.

After six infiltration steps, approximately 10% of the soluble β-1,3-glucanase and up to 5% of the soluble chitinase in the leaf was released into the washing fluid, but only 0.08% of the MDH activity.

Using the azocollase test no proteolytic activity was found in the IWF.

Chromatography on Sephadex G-75. IWF and homogenate were run under the same conditions (Fig. 1, a and b). The use of different salt concentrations in the buffer (either 0.1 M or 0.5 M NaCl) gave the same result. The different salt concentrations were used to prevent nonspecific adsorption of the enzymes to the carbohydrate matrix of the Sephadex material. With IWF, we recovered 75% of the total β-1,3-glucanase activity which eluted in peaks I and II. The M_r of peak I was estimated to be 52 kD and that of peak II had a maximum of 31.3 kD. The IWFs obtained from winter- and summer-grown plants showed differences. Peak I had higher activity during winter time. From December to February, it accounted for 25% of the total recovered activity, but from July to August, for only 8% or less. With the homogenate, approximately 90% of the recovered β-1,3-glucanase activity eluted in a single 20-kD fraction (Fig. 1b).

We used range b in Figure 1b to estimate the amount of extracellular soluble β-1,3-glucanase. Approximately 33% of the β-1,3-glucanase in the IWF eluted in this region, but only 5% (two experiments) was found when the homogenate of noninfiltrated leaves was separated. After threefold infiltration the homogenate contained only 2% (two experiments) of total β-1,3-glucanase activity in this range.

Range d was used to estimate the amount of intracellular β-1,3-glucanase in the IWF. Approximately 45% of the recovered enzyme activity from the homogenate eluted in this range, but from the IWF, only 3% (six experiments) activity was found.

The chitinase activity of the IWF eluted in the same volume as the β-1,3-glucanase peak II, range c. We obtained poorly resolved peaks with a maximum at about 30 kD. Separation of the homogenate led to one peak with a maximum at 29.8 kD.

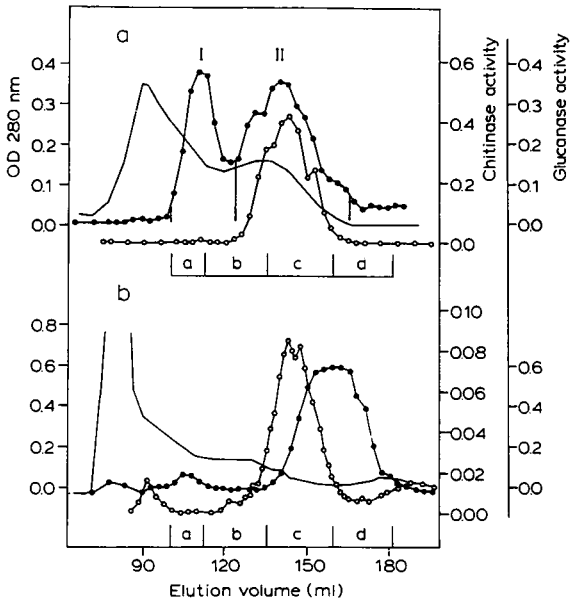

FIG. 1. Gel filtration chromatography on Sephadex G-75 (a) of IWF and (b) of homogenate. The column (2.2 × 80 cm) was equilibrated and eluted with 100 mM sodium-phosphate buffer (pH 7) containing 0.1 M NaCl. Fractions (3.0 ml) were collected at a flow rate of 9.0 ml/h. (●—●), β-1,3-glucanase-activity; (○—○) chitinase activity; (——) A_{280}.

Chromatofocusing. The separation of IWF by chromatofocusing is shown in Figure 2, a and c. Approximately 56% of β-1,3-glucanase and 20% of chitinase activity was recovered. The IWF of leaves infiltrated in summer contained four β-1,3-glucanases, peaks a, c, d, and e. In winter, the IWF contained an additional peak, b (Fig. 2a, insert), and peak c was smaller or missing. Four chitinases, peaks w, x, y, and z, were obtained both in summer and winter IWFs.

Figure 2, b and d, shows the separation of the homogenate where approximately 43% of β-1,3-glucanase and 35% of chitinase activity could be recovered. Of β-1,3-glucanase, 81% eluted in peak f, corresponding to peak a in Figure 2a, which contained approximately 50% of recovered activity. The rest of the β-1,3-glucanase activity eluted in three small peaks: g had no counterpart in the IWF, h eluted in the same pH range as c, and i in the same range as d and e. The chitinase peak x was broader in the homogenate than in the IWF, and peaks y and z were relatively smaller than in the IWF.

Furthermore, the radiometric assay was used to determine if single chitinase fractions are endo-enyzmes. Using this assay, no further isozymes were detected. Both in IWF and homogenate, peak w showed endo- and exo-activity, while peaks y and z exhibited only exo-activity. The case was different with peak x which showed additional endo-activity only in the IWF.

Chromatography on Whatman CM52. For a further characterization of peaks f and a from chromatofocusing, they were chromatographed on Whatman CM52. Each led to one major peak eluting in the same range (Fig. 3). The major peak obtained from f was rechromatographed on Sephadex G-75 and eluted at approximately 21 kD (results not shown), as did the main β-1,3-glucanase activity when homogenate was directly run on Sephadex G-75 (Fig. 1b).

Number of β-1,3-glucanases. In order to find out if peaks I and II of the Sephadex G-75 separation are composed of one or more enzymes, these peaks were chromatofocused. Chromatofocusing showed that peak I (winter only) produced mainly peak

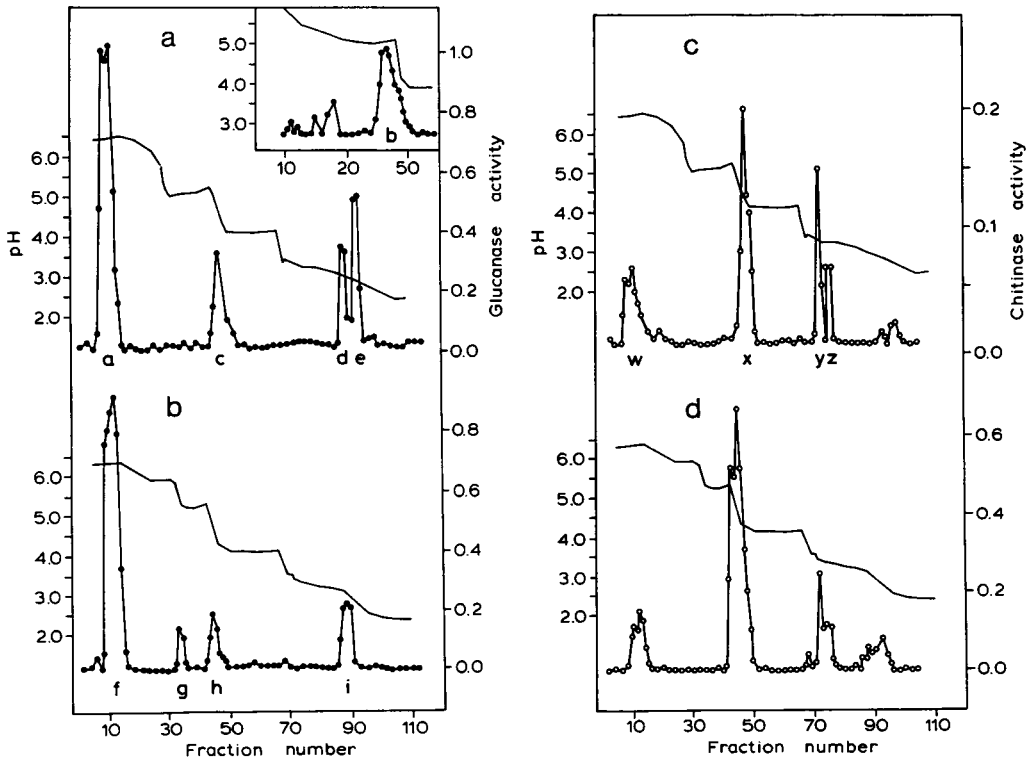

FIG. 2. Chromatofocusing on DEAE-Si500 0.02 to 0.04 mm (Serva) of (a,c) IWF, insert shows chromatofocusing of β-1,3-glucanase peak I after Sephadex G75; (b, d) homogenate. The column (0.8 × 23 cm) was equilibrated with 25 mM histidine/HCl (pH 6.2) and eluted with Servalyt 0.2%, pH 4 (93 mL, flow 13.3 mL/h, fraction size 1.55 mL), followed by Servalyt 0.2%, pH 3.5 (21.6 mL, flow 8.2 mL/h, fraction size 1.35 mL) and Servalyt 0.1%, pH 2.2 (60 mL, flow 8.2 mL/h, fraction size 1.35 mL). Washing with 1 M NaCl in starting buffer did not elute more activity. (●——●) β-1,3-glucanase activity, (○——○) chitinase activity.

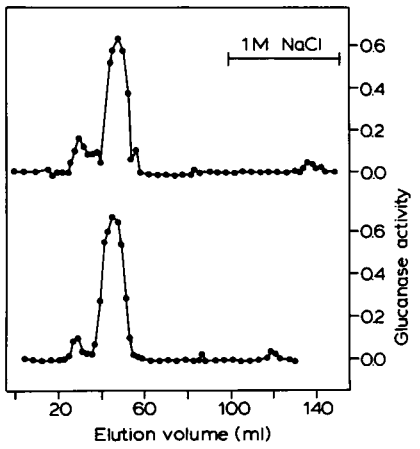

FIG. 3. CM-cellulose chromatography on Whatman CM52 of peak a from chromatofocusing (top) and peak f (bottom). The column (2.2 × 16 cm) was equilibrated and eluted with 50 mM Na-citrate (pH 5.5). Fractions (2.1 mL) were collected at a flow rate of 6.3 mL/h. Washing with 1 M NaCl in elution buffer did not elute more activity. (●——●) β-1,3-glucanase activity.

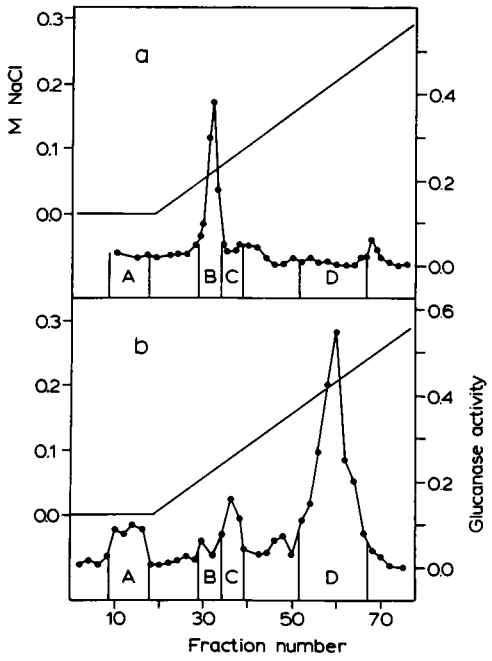

FIG. 4. DEAE-cellulose chromatography on Whatman DE52 (a) of peak I from Sephadex G75 and (b) of peak II. The column (2.2 × 10.5 cm) was equilibrated with 50 mM Tris/HCl (pH 7.5). After loading the sample the column was washed with 37.5 ml of the equilibration buffer. Fractions (2.2 mL) were collected at a flow rate of 4.4 mL/h. The column was then eluted with a linear gradient of 0 to 0.4 M NaCl in 50 mM Tris/HCl (pH 7.5). The total volume of the gradient was 260 ml. Fractions (2.45 ml) were collected at a flow rate of 14.7 mL/h. (●——●) β-1,3-glucanase activity.

b (Fig. 2a, insert); peak II led to the peaks a, c, d, and e. A similar result could be obtained by separating peak I and II on Whatman DE52 (Fig. 4, a and b). The higher mol wt peak (I) corresponded to peak B. Thus, two different chromatographic systems both yielded one peak. The lower mol wt peak (II) led to peaks A, C, and D.

HPLC. While chromatofocusing produced five peaks, Whatman DE52 separation produced only four peaks. Therefore, HPLC anion exchange chromatography was performed on the latter fractions in order to further resolve the peaks. With this method, peak D led to two major peaks with maxima in fractions 19 and 22 (Fig. 5).

SDS-PAGE. As SDS-PAGE (Fig. 6) shows, some of the enzymes were purified to a substantial degree after two purification steps.

Isoelectric Point. The isoelectric points of the various isozymes of IWF and homogenate were estimated from the chromatofocusing elution patterns. Of the IWF peaks, one β-1,3-glucanase and one chitinase (a and w) eluted in the void volume at an isoelectric point higher than 6. The other β-1,3-glucanase peaks eluted in the gradient at approximately pH 5.1 (b), pH 4.9 (c), pH 3.0 (d), and pH 2.9 (e) and the chitinases at pH 4.5 (x), pH 3.3 (y), and pH 3.2 (z). In the homogenate, the main β-1,3-glucanase peak, f, eluted in the void volume, whereas the three minor peaks eluted at pH 5.5 (g), pH 5.0 (h), and pH 2.9 (i). The main chitinase peak eluted in a broad range with two maxima at pH 4.9 and 4.6, whereas the minor chitinase peaks eluted at the same pH as those in the IWF.

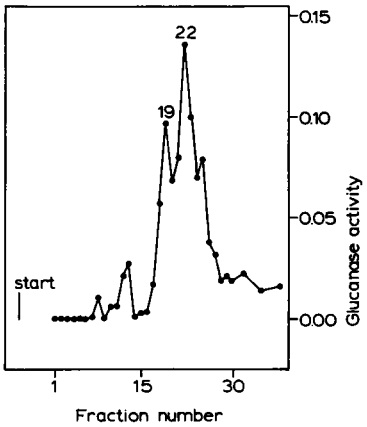

FIG. 5. HPLC on Serva DEAE-Si300, 5 μm, of peak D from Whatman DE52. The column (4.6 × 250 mm) was equilibrated and eluted with 50 mM Na-citrate buffer (pH 5.5). Fractions (0.2 mL) were collected at a flow rate of 0.4 mL/min. Washing with 1 M NaCl in elution buffer did not elute more activity. (●——●) β-1,3-glucanase activity.

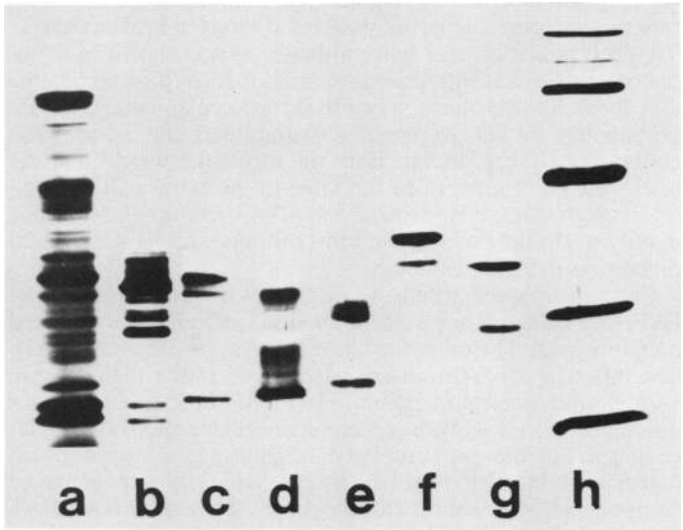

FIG. 6. SDS-PAGE of single enzyme fractions after separation of IWF on Sephadex G-75 followed by chromatofocusing. (a) IWF, (b) peak a/w, (c) peak c/x, (d) peak y, (e) peak z, (f) peak d, (g) peak e, and (h) M_r standards with phosphorylase B (92.5 kD), BSA (67 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), and soybean trypsin inhibitor (21.5 kD).

Action Patterns of β-1,3-Glucanases. Using laminarin as substrate, each of the β-1,3-glucanases, a,b,c,d and e (see Fig. 2) led to different spot patterns with TLC. The β-1,3-glucanases a,c,d, and e produced several spots, indicating that they possess endo-activity; whereas, β-1,3-glucanase b liberated only one spot, which had the same R_F-value as glucose.

DISCUSSION

To be effective in a biotrophic host-parasite interaction during early infection stages, chitinase and β-1,3-glucanase should be present in the cell wall and/or the intercellular space. We found that part of the chitinase and β-1,3-glucanase activity of the oat leaf is located extracellularly before fungal infection. With sixfold infiltration, we released about 5% of chitinase and about 10% of β-1,3-glucanase activity of the whole leaf. A further estimation for the extracellular amount of β-1,3-glucanase came from separation on Sephadex G-75. By comparison of a range with low activity in the homogenate but high activity in the IWF (range b in Fig. 1), we calculated that about 15% of the total activity is extracellular. Thus, a considerable amount of enzyme is present in the cell wall. But it should be taken into consideration that, with the infiltration method, only soluble wall chitinases and β-1,3-glucanases can be recovered and not any covalently bound ones, which could also act against pathogens.

Our results show that the infiltration technique used in this study is suitable for extracting enzymes specifically from the intercellular space, without major contamination from the cytoplasm. The degree of purity of the IWF was quantified by using the marker enzyme MDH (21). MDH activity was the same as found for IWF from barley leaves (21). The infiltration method used was so gentle that the MDH activity released was not increased in consecutive infiltration steps. Comparison of the β-1,3-glucanase and chitinase activity with that of the MDH showed that these enzymes were enriched in the IWF at least 60-fold to MDH. Further evidence for the high purity of the IWF was achieved by comparing the elution profiles of β-1,3-glucanase on Sephadex G-75 in a range where there is only low activity in the IWF but very high activity in the homogenate (range d, Fig. 1). Assuming that, in the worst case, all activity here is of intracellular origin, only 6% of the β-1,3-glucanase contained in the IWF would be intracellular contamination. These data indicate that measured activities were not a result of broken cells.

IWF contains at least four chitinases as was shown by chromatofocusing. Gel filtration gave only a single peak indicating that these enzymes have very similar molecular weights of approximately 30 kD. Apparently, extracellular and intracellular chitinases are very similar. Both the molecular weight and the isoelectric point seem to be the same in the extra- and intracellular isozymes. Only one isozyme at pH 4.9 seems to be confined mainly to the cell interior, and the chitinases at pH 4.5 showed differences in endo-/exo-activity.

Chromatographic results show five β-1,3-glucanases in the IWF. One of them has a M_r of about 52 kD and was observed only in winter. The other four have M_r of approximately 31 kD. The different β-1,3-glucanases outside and inside the cell have very similar isoelectric points. However, there is an obvious difference in mol wt between the extracellular and intracellular enzymes, *i.e.* the extracellular β-1,3-glucanases have a much higher mol wt. This might be due to addition of noncharged components, *e.g.* neutral carbohydrates. Miyata and Akazawa (15) show that the isoelectric point of a protein might be changed only little when carbohydrates are added. This additional component could be a signal for transport out of the cell.

As both enzymes are preexistent in the plant cell wall, they could have a function during pathogenesis. They could act as a preformed nonspecific defense barrier against a wide range of pathogens by degrading their cell wall.

On the other hand, rather than involving direct attack on the fungal walls, these enzymes may mediate recognition by releasing defined oligosaccharides from the fungal surface. One interesting possibility would be the triggering of the fungal infection structures, *e.g.* the haustorial mother cell of a rust fungus. These enzymes may also mediate host responses. On one hand chitinase and β-1,3-glucanase can release elicitors of lignification or phytoalexin accumulation (9, 10), while on the other hand, released oligosaccharides could act as suppressors of phytoalexin accumulation (28). Specific plant hydrolases also act in the symbiosis of *Rhizobia* species. The plant hydrolases alter the surface carbohydrates of the respective symbiont in a different way from those of the nonsymbiont, although the carbohydrate composition is nearly the same (24).

We think that in our case extracellular chitinases and β-1,3-glucanases could have specific functions. The high number of isozymes and their different mode of action could imply a specificity of the different enzymes regarding their ability of liberating saccharides. This was shown for two β-1,3-glucanase isozymes isolated from pea (27).

We will study the role of the different isozymes in compatible and incompatible interactions between oat and oat crown rust and after unspecific stress. However, we should not narrow our view by assuming that the β-1,3-glucanases in the cell wall of oat leaves may have a major role only during pathogenesis. The finding that one isozyme was observed only during winter conditions suggests that there are other unknown tasks of these enzymes in the physiology of the leaf.

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