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Peroxidases and Glycosidases in Intercellular Fluids from Noninoculated and Rust-Affected Wheat Leaves[1]

ISOZYME ASSAY ON NITROCELLULOSE BLOTS FROM TWO-DIMENSIONAL GELS

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ABSTRACT

Proteins in intercellular washing fluid (IWF) from noninoculated and stem rust-affected wheat leaves were separated by isoelectric focusing and polyacrylamide gel electrophoresis under nondenaturing conditions, transferred to nitrocellulose membranes, and assayed *in situ* for peroxidase and glycosidase activity.

Two infection-related peroxidase isozymes were detected in addition to more than ten that were present only in noninoculated plants. One other peroxidase isozyme was present in much higher concentration in IWF from infected leaves than in IWF from noninoculated leaves.

IWF contained many polymorphic glycosidases. A new method is described to localize the glycosidase isozymes accurately on nitrocellulose blots for evaluation of their substrate specificities: each blot was developed with the appropriate *p*-nitrophenyl glycoside to reveal glycosidase activity, then reprobed for concanavalin A-binding glycoproteins to serve as an internal reference frame for blot-to-blot comparisons. This procedure also provided information on glycosylation of the isozymes.

The locations of at least 15 (out of 37) isozymes were coincident with concanavalin A binding, including those of all 10 α-D-mannosidases, and of 6 β-D-xylosidases. On five areas of the blots there was coincidence of β-D-xylosidase and α-L-arabinosidase activity. Three of these areas corresponded to three of the most prominent Coomassie brilliant blue-stainable IWF proteins in gels. Isozymes that could convert *p*-nitrophenyl-β-D-glucoside, -β-D-galactoside, and/or -β-D-fucoside revealed a complex pattern of partially overlapping substrate specificities: two isozymes utilized both glucose and fucose derivatives, one utilized all three derivatives, and several others converted only one of the three substrates. No enzymes were detected with activity on *p*-nitrophenyl-β-D-galactosaminide, -β-L-fucoside, or -α-D-galactoside. No additional glycosidases were detected in IWF from stem rust-affected leaves.

Previous studies on the composition of intercellular washing fluid from primary leaves of wheat and barley (11, 14) demonstrated that the majority of proteins detected in this fluid probably were of extracellular origin and were not cytoplasmic contaminants from injured cells. IWF[3] from stem rust- or leaf rust-affected leaves of wheat contained 'infection related' proteins, many of which were glycosylated (11). These may have originated from the surfaces of fungal intercellular hyphae. We were interested to know what enzyme activities are associated with proteins in IWF from noninoculated and infected wheat. Cell wall-associated enzymes from various plant tissues have been investigated by others, using intercellular fluids (1, 8, 13, 17). Use of this fluid avoids losing weakly bound, buffer-soluble proteins, a problem encountered when plant cell walls are purified from homogenates by conventional means (3).

In our previous study (11), IWF proteins were separated by two-dimensional IEF-PAGE under nondenaturing conditions, and transferred to nitrocellulose membranes for glycoprotein analysis. This method is also useful for the study of enzyme polymorphism, but is limited to those enzymes for which substrates are available that yield visible, insoluble products. Other characteristics of the immobilized isozymes can be determined by reprobing the same blot with other reagents, such as lectins. We have used Con A as a second probe in this study to reveal glycoproteins. These served as internal standards for comparisons between blots exposed to different substrates, and for evidence of glycosylation of some glycosidases in IWF.

MATERIALS AND METHODS

The growth of wheat (*Triticum aestivum* L. cv Little Club) at 19°C, infection with the stem rust fungus (*Puccinia graminis* f. sp. *tritici* Eriks, and E. Henn., race [36(48)]) and preparation of IWF from noninoculated wheat leaves or from wheat leaves containing 4-d-old infections of stem rust were done as previously reported (11).

Spot tests for enzyme activity were conducted using samples of IWF containing ~3 μg or ~8 μg protein (from noninoculated or infected leaves, respectively), since the protein yield from infected leaves was approximately twice that of noninoculated leaves. Samples were applied to nitrocellulose membranes and incubated for 30 min with *p*-nitrophenyl glycoside (1 mg/ml in 0.2 M Na acetate buffer [pH 5.2]). *p*-Nitrophenyl-*N*-acetyl-β-D-galactosaminide (2-acetamido-2-deoxy-β-D-galactopyranoside), -α-L-arabinofuranoside, -β-D-fucopyranoside, -β-L-fucopyranoside, -α-D-galactopyranoside, -β-D-galactopyranoside, -β-D-glucopyranoside, -α-D-mannopyranoside, and -β-D-xylopyranoside were purchased from Sigma. In some experiments, the *p*-nitrophenyl glycoside solution also contained 2.5 mM D-glucono-1,5-lactone (Sigma). To reveal *p*-nitrophenol, membranes were air-dried and sprayed with 1 M NH_3 solution, containing 2 mM EDTA.

Two-dimensional gel electrophoresis of IWF proteins by IEF-PAGE under nondenaturing conditions, staining with CBB, or protein transfer to nitrocellulose membranes and assay with *p*-

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[3] Abbreviations: IWF, intercellular washing fluid; IEF, isoelectric focusing; Con A, concanavalin A; CBB, Coomassie brilliant blue; TBS, Tris-buffered saline.

nitrophenyl glycosides were done as previously described (11). For each experiment, 175 μg protein was applied to the IEF gel. After marking centers of enzyme activity with small holes, blots were photographed using a blue filter, washed with double-distilled H_2O, dried, autoclaved (121°C, 15 min), and probed with Con A and horseradish peroxidase to reveal glycoproteins (15).

To detect endogenous peroxidases, blots were equilibrated for 15 min with TBS following their removal from the gels, and then exposed to a solution of 0.06% 4-chloro-1-naphthol, 0.01% H_2O_2 in TBS (10) for 2 min. Blots were washed with double-distilled H_2O and air-dried.

All experiments were repeated at least twice, using fresh plant material.

RESULTS

Two infection-related isozymes of peroxidase in IWF (open arrowheads, Fig. 1B) were detected reproducibly on nitrocellulose membranes in addition to more than ten that were present only in noninoculated plants (Fig. 1A). Traces of an additonal isozyme were detected in a replicated experiment in IWF from noninoculated wheat leaves (result not shown), but the isozyme was present at a much higher concentration in IWF from infected leaves (closed arrowhead, Fig. 1B). Other peroxidase isozyme differences between noninoculated and infected leaves in Figure 1 were not reproducible.

Spot tests for glycosidases in IWF gave no evidence for the presence of enzymes with activity on *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide, -β-L-fucoside or -α-D-galactoside. However, glycohydrolytic activity was detected when spot tests were done with *p*-nitrophenyl-α-L-arabinoside, -β-D-fucoside, -β-D-galactoside, -β-D-glucoside, -α-D-mannoside, and -β-D-xyloside. Identical results were obtained using IWF from noninoculated or infected leaves. D-Glucono-1,5-lactone inhibited enzymic hydrolysis of *p*-nitrophenyl-β-D-glucoside, reduced hydrolysis of *p*-nitrophenyl-β-D-fucoside, but did not affect conversion of *p*-nitrophenyl-β-D-galactoside.

Substrates which gave positive results in spot assays were incubated with IWF proteins from noninoculated and infected leaves that had been separated by two-dimensional electrophoresis and transferred to nitrocellulose membranes. No reproducible differences could be found between the isozyme patterns of IWF from noninoculated wheat and those from infected wheat with any of the substrates tested. Because glycosidase isozymes are known to vary in their substrate specificity, and since there was slight run-to-run variation caused by sample-generated alteration of the pH gradient in IEF gels, it was helpful to have an internal standard for reliable comparisons between blots. In a previous study (11), the positions of host isozymes with β-D-fucosidase activity were used to compare glycoprotein patterns of IWF from two different sources. In the present study, the endogenous Con A-binding proteins provided the frame of reference: blots which had been used to detect glycosidase activity were postdeveloped to reveal Con A-binding proteins. For example, Figure 2, A and B, shows the same blot which had been first assayed with *p*-nitrophenyl-β-D-xyloside as substrate, and then reprobed with Con A. The centers of activity of six isozymes were coincident with centers of activity of the probe used (horseradish peroxidase) to reveal Con A-binding proteins. A seventh isozyme (arrowhead, Fig. 2B) was not coincident with Con A binding. The enzyme pattern was the same on blots made from gels in which IWF proteins from infected leaves had been separated. This pattern, relative to host and infection-related glycoproteins, is shown in Figure 2C. A typical CBB-stained gel in which IWF proteins from noninoculated wheat were separated is illustrated in Figure 2D. Three dominant proteins (arrows) occupied similar positions as those of three isozymes with xylosidase activity on blots.

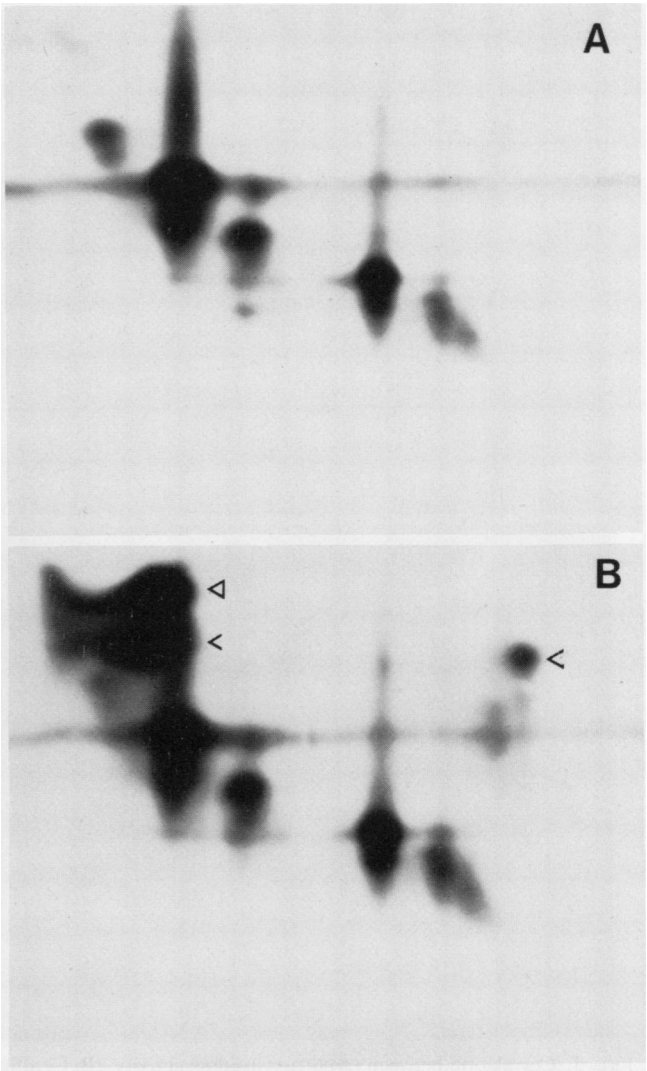

FIG. 1. Endogenous peroxidase isozymes in IWF from (A) noninoculated and (B) stem rust-affected wheat leaves harvested 4 d after infection. Proteins were separated by IEF from cathode (left) to anode (right) followed by PAGE (top to bottom) under nondenaturing conditons, transferred to nitrocellulose membranes, and developed with 4-chloro-1-naphthol and H_2O_2. Figures are representative of entire gels. B, two infection-related isozymes (<) and one isozyme that was present in much higher concentration in IWF from infected plants (◁).

Locations of isozyme activity on the six substrates that were positive in spot assays, relative to the pattern of reproducibly detectable Con A-binding proteins from noninoculated leaves, are illustrated schematically in Figure 3 and summarized in Table I.

DISCUSSION

The question of contamination of IWF with intracellular proteins has been dealt with at length in previous studies (11, 14). However, the possibility that these fluids may be contaminated with constituents of vascular tissue has not, to our knowledge, received much attention. We have obtained IWF from infiltrated leaves which had been centrifuged with the leaf tips directed towards the base of the centrifuge tube in an attempt to prevent constituents of the sieve tubes from being released into IWF; the protein and glycoprotein patterns from this fluid were indistinguishable from those illustrated in this paper (D. W.

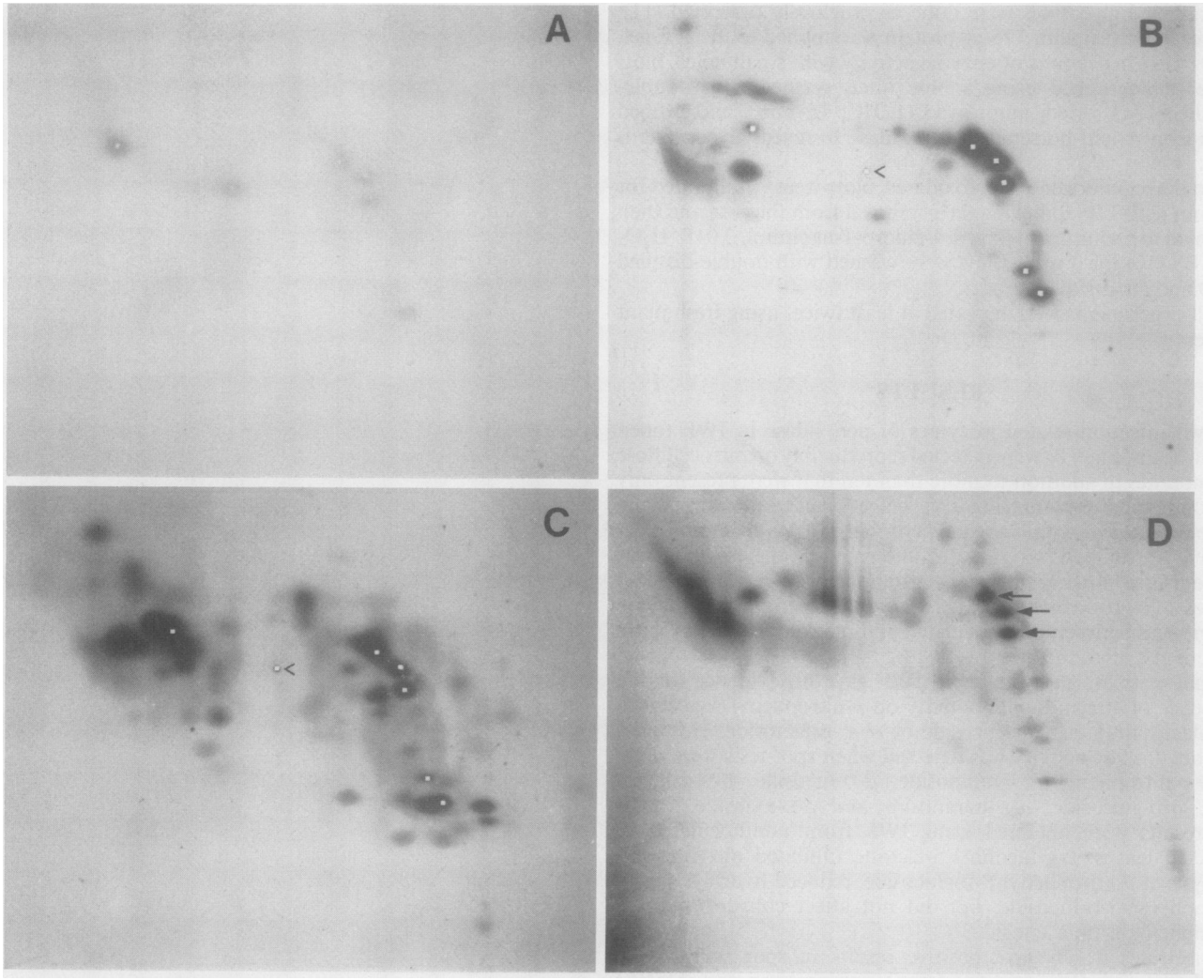

FIG. 2. Correlation between (A) β-D-xylosidase activity, (B, C) affinity to Con A, and (D) stainability with CBB of proteins from wheat leaf IWF separated as in Figure 1. Nitrocellulose blot with IWF proteins from noninoculated leaves was first developed for β-D-xylosidase activity (A), then reprobed for Con A-binding glycoproteins (B). (□) centers of enzyme activity; (<), points to xylosidase activity that was not coincident with Con A binding. The same xylosidase isozymes were detected in IWF from stem rust-affected wheat (C) among a greater number of Con A-binding glycoproteins. A CBB-stained gel with IWF proteins from noninoculated leaves is shown in D; three major proteins (arrows) occupy similar positions on the gel as three of the seven xylosidases on blots (compare B with D).

Holden, R. Rohringer, unpublished data). This, together with the evidence that the major IWF proteins are typical cell wall-associated glycosidases, leads us to conclude that proteinaceous contaminants from vascular tissue probably do not constitute a large proportion of the total in IWF.

The pattern of Con A-binding proteins in IWF from plants grown at 19°C was reproducible and served as a frame of reference to localize enzyme activity. The reproducibility of glycoprotein detection indicates that alteration of glycoproteins by cleavage of exposed, terminal glucoside and mannoside residues by endogenous enzymes could not have been significant. It should be noted that there were more Con A-binding proteins in IWF grown under these conditions than were detected in a previous study (11) where some plants were grown under conditions which resulted in greater contamination of IWF with cytoplasmic proteins.

The large number of peroxidase and glycosidase isozymes that was detected in IWF reflects the resolving power of two-dimensional separations of native proteins by IEF-PAGE. It was not possible to determine if the peroxidases that were detected had affinity to Con A, because the colored product that indicated endogenous enzyme could not be erased prior to probing with Con A and horseradish peroxidase. The presence of infection-related peroxidases in intercellular fluid appears to be inconsistent with the findings of Seevers *et al.* (16), who could not detect qualitative differences in peroxidases between noninoculated and stem rust-affected leaves of susceptible wheat. However, we separated IWF proteins using a two-dimensional technique, whereas they used PAGE for proteins of whole leaf homogenates from a different cultivar and detected activity with a different substrate. It is not known whether the infection-related peroxidases that we detected were of host or fungal origin.

Most of the xylosidase and arabinosidase activity that we detected appeared to reside in three glycoproteins (isozymes 31–33; Figs. 2 and 3) that constituted a major proportion of total IWF protein. The natural substrates for these enzymes could include xylan, xyloglucan, and glucuronoarabinoxylan, among others (5). The coincidence of xylosidase and arabinosidase activity in five areas on the blots (isozymes 30–34; Fig. 3) is surprising since the arabinoside and xyloside substrates were in the furan and pyran forms, respectively. To our knowledge, activity of a single enzyme on both types of glycosides has not

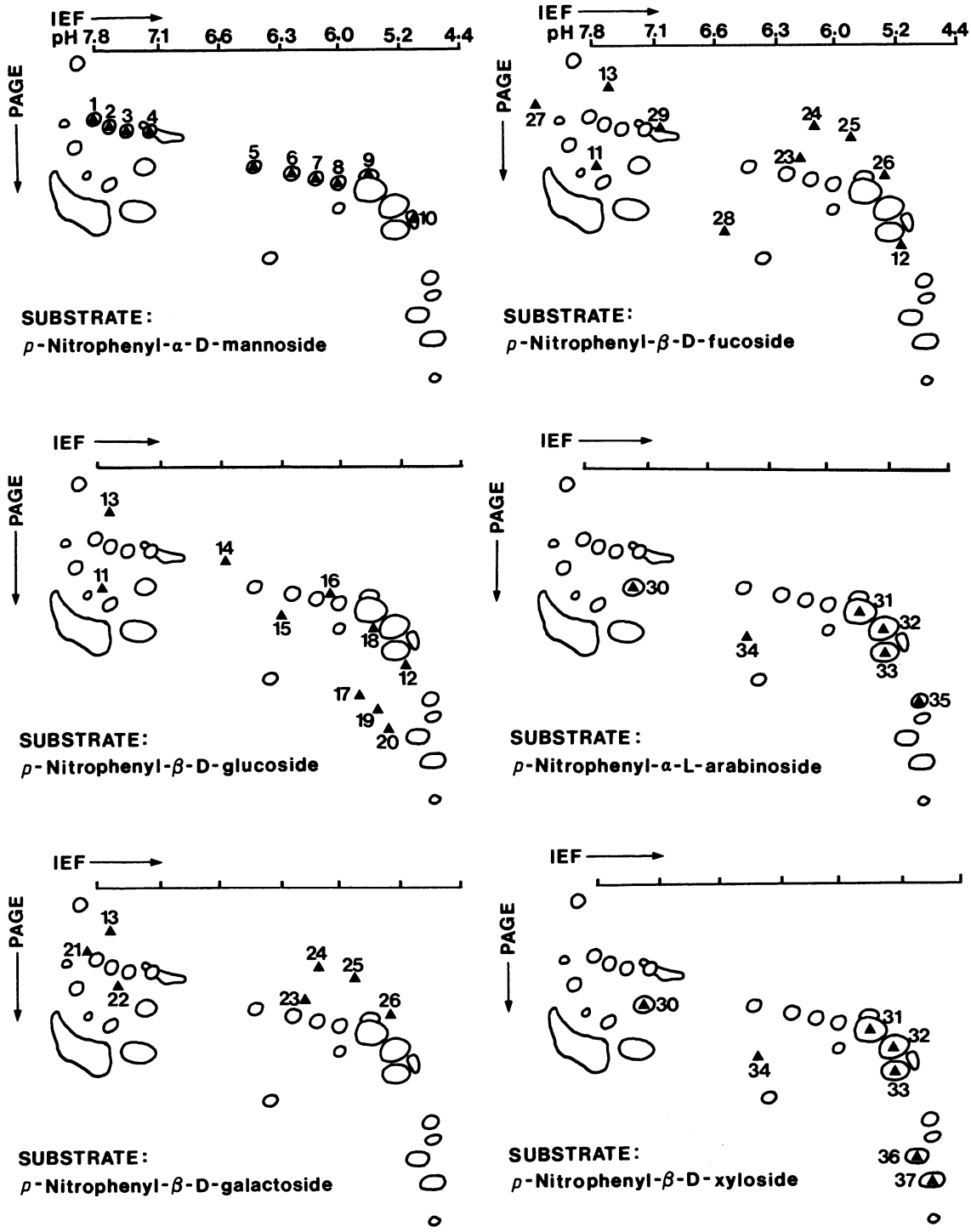

FIG. 3. Polymorphic glycosidases in IWF from noninoculated wheat leaves. Proteins in IWF were separated as in Figure 1 and transferred to nitrocellulose membranes; these were incubated with a solution containing the appropriate *p*-nitrophenyl glycoside, then reprobed to reveal Con A-binding glycoproteins (encircled areas). Solid triangles on the diagrams indicate location of enzyme activity on blots. Numbered isozymes are summarized in Table I. Note overlapping substrate specificities and coincidence with Con A binding of some of the isozymes.

been reported. There are at least four possible explanations for this observation. First, hydrolytic activity on both types of substrates could have been due to comigration of an arabinosidase and a xylosidase to each of the five regions of activity; since each of these 'paired' isozymes would have to share both molecular size and charge characteristics, this would be an unlikely coincidence. Second, lectin-mediated aggregation between glycosylated arabinosidases and xylosidases may have occurred and the aggregates may have survived the conditions of separation; although lectin properties have been ascribed to α-galactosidases (7), invoking such a mechanism to explain our results would require additional suppositions to account for the single area of arabinosidase/xylosidase activity (isozyme 34; Fig. 3) that was not coincident with Con A binding. Third, some of the isozymes may consist of heterodimers or other aggregates of subunits; this hypothesis is not consistent with the glycosylation pattern of isozymes 30 to 37 (last column, Table I) and would require the presence of subunits in IWF not detected in our assays. Finally,

Table I. *Substrate Specificities and Presumed Con A-binding Properties of Glycosidase Isozymes in Intercellular Washing Fluid*

Isozyme No. (from Fig. 3)	*p*-Nitrophenyl Derivative Converted	Was Isozyme Location on Blots Coincident with Con A-binding?
1,2,3,4,5,6,7,8,9,10	α-D-Man	Yes
11,12	β-D-Glc, β-D-Fuc	No
13	β-D-Glc, β-D-Fuc, β-D-Gal	No
14,15,16,17,18,19,20	β-D-Glc	No
21,22	β-D-Gal	No
23,24,25,26	β-D-Gal, β-D-Fuc	No
27,28	β-D-Fuc	No
29	β-D-Fuc	?[a]
30,31,32,33	α-L-Ara, β-D-Xyl	Yes
34	α-L-Ara, β-D-Xyl	No
35	α-L-Ara	Yes
36,37	β-D-Xyl	Yes

[a] Coincidence of isozyme and Con A binding was equivocal.

it is possible that hydrolysis of both substrates is catalyzed by the same enzyme; further attempts to separate the two activities would be required to demonstrate this conclusively.

The results with isozymes possessing activity on *p*-nitrophenyl-β-D-glucoside, -β-D-galactoside, and/or -β-D-fucoside revealed a complex pattern of partially overlapping substrate specificities. Activity on the fucoside has been attributed to a secondary activity of galactosidases (6); while this could explain some of our observations (isozymes 23–26), we found two isozymes which could convert the glucose and fucose derivatives, and one isozyme that could convert all three, in addition to several that could utilize only one of the three substrates (Table I). It may be significant that none of the positions of these isozymes, with the possible exception of one, was coincident with affinity to Con A. Overlapping substrate specificities of these types of isozymes have been reported in barley (4). The ability of D-gluconolactone to diminish but not inhibit conversion of the fucoside in spot tests can be explained by the presence of some fucosidase isozymes (11–13 in Fig. 3) that were capable of utilizing the glucoside, and others that were not (isozymes 23–29).

In contrast to the isozymes discussed above, none of those utilizing the mannose derivative had activity on any other substrate used, and all of them had affinity to Con A. Periodate-Schiff reaction indicated that the α-mannosidase purified by Greve and Ordin (9) from oat coleoptiles was also glycosylated. However, the enzyme they described was tightly associated with the cell wall, and required 3 M LiCl to be solubilized.

At least 15 of the 37 glycosidase isozymes were coincident on blots with hapten-inhibitable Con A affinity. We conclude from this that these enzymes are glycosylated, as predicted by Lamport (12). The remaining isozymes lack affinity to Con A, but they may contain sugars other than mannose or glucose. Although the principal reason for autoclaving blots prior to probing with Con A was to inactivate endogenous peroxidases, this may be an important pretreatment for the detection of some glycosylated enzymes, because Bowles *et al.* (2) showed that the mannosyl residues of jackbean α-D-mannosidase are sterically masked from interacting with Con A unless the enzyme is denatured.

Some glycosidases in IWF may have escaped detection if their pH optima are far removed from that used in the assays (pH 5.2) or if they have pI values outside the range (pH ~4 to ~8) used by us for IEF. That α-D-galactosidase activity was not detected in our spot tests is surprising, considering the wide distribution of this enzyme in the plant kingdom and the fact that it is generally found in the soluble fraction (6) and associated with cell walls (18). Most of the plant α-galactosidases investigated have pH optima between 5 and 6 (6).

We are not aware of any reports on exoglycosidases produced by rust fungi. Under the conditions of our assay we found no evidence for fungal glycosidases, since no additional isozymes were detected in IWF from infected plants. It is interesting that most Con A-binding glycoproteins in IWF from noninoculated wheat leaves apparently are glycosidases, while the more than 20 that are presumed to originate from the fungus (11) in stem rust-affected leaves evidently are not. It is not known if any enzyme activity is associated with the fungal glycoproteins in this tissue, or what role they may have in the host-parasite interaction.

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