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Activation of *Avena* Coleoptile Cell Wall Glycosidases by Hydrogen Ions and Auxin[1]

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ABSTRACT

Several cell wall-bound glycosidases present in *Avena sativa* coleoptiles were assayed by following the hydrolysis of *p*-nitrophenyl-glycosides. Particular emphasis was placed on characterizing some parameters affecting the activity of β-galactosidase. The pH optimum of this enzyme is 4.5 to 5.5; it is sensitive to copper ions and *p*-chloromercuribenzoate treatment and apparently has an exceptionally low turnover rate. Indoleacetic acid treatment enhanced *in vivo* β-galactosidase activity of coleoptile segments by 36% over control after 60 minutes. This enhancement was prevented by abscisic acid and cycloheximide. High buffer strengths and low pH reduced the indoleacetic acid-enhanced increase in enzyme activity. These data lend support to the following proposed model of indoleacetic acid action. Indoleacetic acid enhances the release of hydrogen ions into the cell wall which promote the activities of cell wall glycosidases, some of which may participate in the cell extension process.

It is well established that both indoleacetic acid and hydrogen ions can elicit rapid growth responses in a number of excised plant tissues (7–9, and references cited therein). In many respects, the H^+-induced extension of *Avena* coleoptiles is similar to that evoked by IAA (7, 9, 23), suggesting that the two growth effectors may be operating by a similar, if not identical, mechanism in the enhancement of cell wall loosening and cell extension. Hager *et al.* (9) presented indirect evidence linking the two growth responses and proposed a model in which IAA acted to stimulate a membrane-bound ATPase or proton pump which would release H^+ into the wall. According to this model, H^+ would accumulate in the cell wall region and initiate cell wall loosening. Recently, direct evidence for IAA-induced H^+ secretion from cells of pea stem segments (17, 18) and *Avena* coleoptiles (4, 22) has been reported. Since the magnitude of auxin-induced H^+ secretion in these tissues is sufficient to increase the rate of cell elongation, at least part of the IAA-induced growth response must be attributed to the increased level of H^+ in the cell wall (see ref. 22 for a full discussion of this argument).

The mechanism by which H^+ stimulate cell elongation is yet unknown. One possibility is that the protons may enhance the activities of cell wall-bound enzymes that hydrolyze polysaccharides (9, 11), a condition which may lead to cell wall loosening. We have conducted some tests with this hypothesis in mind, and this paper relates substantial correlative evidence that at least the activity of one cell wall-bound glycosidase (a β-galactosidase) behaves as if it were involved in IAA-induced cell elongation.

MATERIALS AND METHODS

Preparation or Seedlings, Segments, and Cell Walls. Seeds of *Avena sativa* L. cv. Victory were surface-sterilized in 1% NaOCl, rinsed, soaked in tap water for 2 hr, and then sown in moist vermiculite in the dark at 20 C. After 5 days, coleoptiles were excised under normal laboratory lighting conditions and placed on ice-cold moist toweling until enough were prepared for the experiment. Nonpeeled segments were prepared by cutting 10-mm sections beginning 3 mm below the apex. Peeled segments consisted of similar 10-mm sections from which the cuticle and epidermis had been physically removed. Cell walls were isolated at 4 C by grinding 15- to 20-mm coleoptile segments in distilled H_2O in a mortar and pestle, then further homogenizing in a glass tissue grinder. Recovery of cell wall-bound glycosidase activity using such a method was no different than that obtained when an 80% (v/v) glycerol extraction technique (12) was employed (Cohen and Johnson, unpublished data). The homogenate was centrifuged at 500*g* for 5 min, and the resulting cell wall pellet was washed three times in 15 volumes of H_2O by resuspension and recentrifugation at 500*g* for 10 min. Further H_2O washes removed only about 3% of the remaining pelletable enzyme activity with each wash and were therefore omitted.

Enzyme Assays. For assays using nonpeeled or peeled segments, 20 nonpeeled or 5 peeled segments were rinsed thoroughly with distilled H_2O and placed into a 25-ml Erlenmeyer flask containing 1.5 ml of phosphate buffer (pH 6.5–7.0) or phosphate-citrate buffer (pH 3.0–6.0), and, unless otherwise noted, allowed to incubate for 30 min at 30 C. After this preincubation period, the assay was initiated by the addition of 0.5 ml of 40 mM *p*-nitrophenyl-β-D-galactopyranoside or other *p*-nitrophenyl-glycoside (Sigma Chemical Co.). Final concentration of the buffer was 10 mM unless otherwise noted. After an additional 30 min at 30 C, the reaction was terminated by the addition of 3 ml of 2% (w/v) Na_2CO_3. The solution was then thoroughly mixed and decanted into colorimeter tubes and its absorbancy read at 415 nm on a Bausch and Lomb Spectronic 20 spectrometer. Although absorbance units are used throughout the text, the amount of *p*-nitrophenol released under these conditions can be calculated from the observed extinction coefficient: 1.0 μmole *p*-nitrophenol = 3.25 A_{415} units. Each datum represents the average of duplicate or triplicate determinations.

For assays of glycosidase activity from cell wall prepara-

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tions, 0.5 ml of the cell wall suspension (wall material from 5–10 coleoptiles/1 ml of suspension) was added to 1.0 ml of 20 mM buffer (variable pH) in a test tube at 30 C. Initiation, termination, and other conditions of the reaction were as stated above. After termination of the reaction, cell walls were removed by centrifugation and the amount of *p*-nitrophenol released was determined from analysis of the supernatant. Each specific treatment was done in duplicate.

RESULTS AND DISCUSSION

Earlier pH profiles for extension of nonpeeled *Avena* coleoptile segments indicates that H^+-induced growth occurs optimally at pH 3.0 (23). More recently, however, it was learned that the coleoptile cuticle presents a greater barrier to H^+ influx than originally suspected. By the simple expedient of physically removing the cuticle and outer epidermis, it was shown that the resultant peeled coleoptiles extended at an optimal rate near pH 5.0 (22). Therefore, any condition which would lower the pH of the cell wall to 5.0 or less would lead to cell extension, presumably through some wall loosening mechanism. This revised pH profile as well as other data suggested to us the possible involvement of cell wall-bound glycosidases in cell elongation, since it has been shown that some of these enzymes exhibit optimal activity ranging between pH 3.0 to 5.5 (1, 6).

Figure 1a shows pH profiles for β-galactosidase activity of *Avena* coleoptiles. For nonpeeled segments, there is a gradual increase in enzyme activity as the pH is lowered to 3.0. It would appear that the cuticle is limiting the level of observable activity, for when the cuticle is removed, the resultant peeled segments exhibit an increased level of β-galactosidase activity. Furthermore, the peeled segments show a definite pH optimum at 4.5 with respect to enzyme activity. We interpret these differences between peeled and nonpeeled segments to indicate that in the former case, a greater cell surface area is available for H^+ and substrate entry, a condition which not only leads to greater observable hydrolysis activity, but may also explain why peeled segments exhibit a pH optimum at 4.5, since a greater proportion of the enzyme population, unshielded by the waxy cuticle, would be exposed to the low pH environment. Isolated cell walls also exhibit a definite pH optimum near 5.0 but have a somewhat lower activity than the peeled segments on a per coleoptile basis. The higher β-galactosidase activity in peeled segments, as opposed to cell walls, may result because some substrate may enter the cytoplasm and become hydrolyzed by soluble β-galactosidase. It is also possible that a fraction of extra-cytoplasmic enzyme may be washed off the cell walls during preparation of the walls. Since less than 5% of the total β-galactosidase activity of peeled sections is due to released enzyme present in the surrounding medium under the conditions of preincubation and assay (unpublished data), and if we assume that the measurable β-galactosidase activities present in peeled segments and cell wall preparations reflect accurately the levels of enzyme complement in each case, then we may deduce that at least half of the activity present in peeled segments is located in the cell walls (Fig. 1a).

If the amount of cut surface area exposed in nonpeeled coleoptile segments is increased by cutting the segments into successively smaller pieces, the amount of observable β-galactosidase activity increases proportionately (Fig. 1b). Apparently, entry of H^+ and *p*-nitrophenyl-β-galactoside occurs predominantly from the cut ends, and this is supported by the visual observation that for any given length of segment, only the end 1 to 1.5 mm of the segments turned yellow upon the addition of Na_2CO_3, indicating that the product *p*-nitrophenol was formed predominantly at or near the cut ends. A concentration gradient of substrate or H^+ or both from the cut surface inward may explain the relatively low pH optimum of 3.0 or less (Fig. 1a) when nonpeeled coleoptiles were assayed.

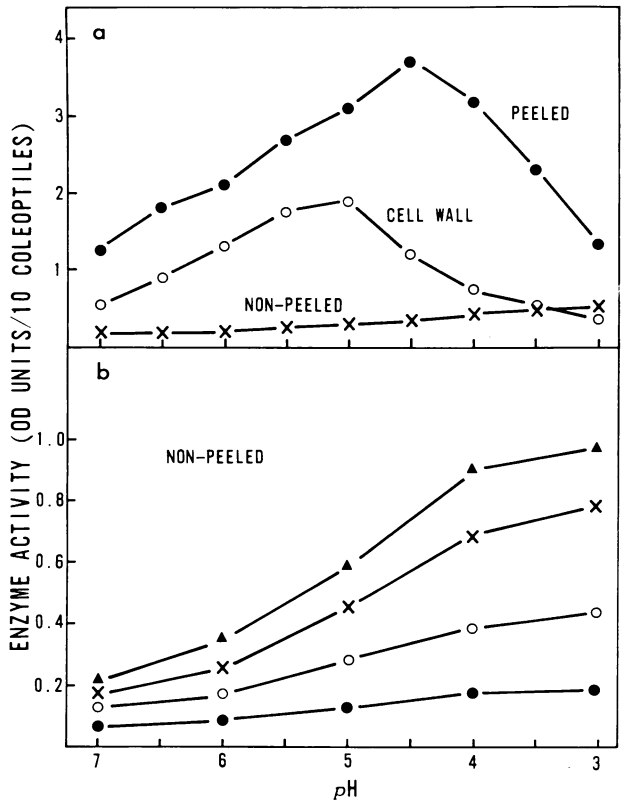

FIG. 1. pH curves for β-galactosidase activity of (a) nonpeeled or peeled coleoptile segments, or segments from which cell walls were prepared; or (b) nonpeeled, nondecapitated segments bearing one cut end (●), decapitated segments with two cut ends (○) or decapitated segments cut into halves or thirds bearing four (×) or six (▲) cut ends, respectively.

Table I. *Effects of p-Chloromercuribenzoate, Cu^{2+}, and HCl Pretreatment of Cell Walls on β-Galactosidase Activity*

Cell walls were incubated 45 min at 30 C in 0.1 M citrate buffer, pH 5.0 with or without 0.1 mM *p*-chloromercuribenzoate or 5 mM $CuSO_4$, then 0.5 ml of 40 mM *p*-nitrophenyl-β-D-galactopyranoside was added for the enzyme assay. For HCl treatment, the cell walls were treated for 45 min at 30 C in 0.1 *N* HCl, then washed twice with 0.1 M citrate buffer, pH 5.0, and assayed as above. The data represent the average of duplicate determinations.

Inhibitor	Enzyme Activity
	absorbance units/10 coleoptiles
None	1.68
p-Chloromercuribenzoate	0.61
Cu^{2+}	0.57
0.1 N HCl pretreatment	0.01

On the basis of a 65% inhibition of pH 4.0-induced growth of plasmolyzed sunflower hypocotyls by 5 mM Cu^{2+}, Hager *et al.* (9) proposed that an enzymatic mechanism was involved in H^+-induced growth. Using frozen-thawed rye coleoptiles, we have confirmed the claim that H^+ extension is sensitive to Cu^{2+} and also found that the sulfhydryl reagent, *p*-chloromercuribenzoate, inhibits H^+-induced extension (Rayle, unpublished data). The activity of *Avena* cell wall β-galactosidase is also sensitive to these reagents (Table I), and this is consistent with

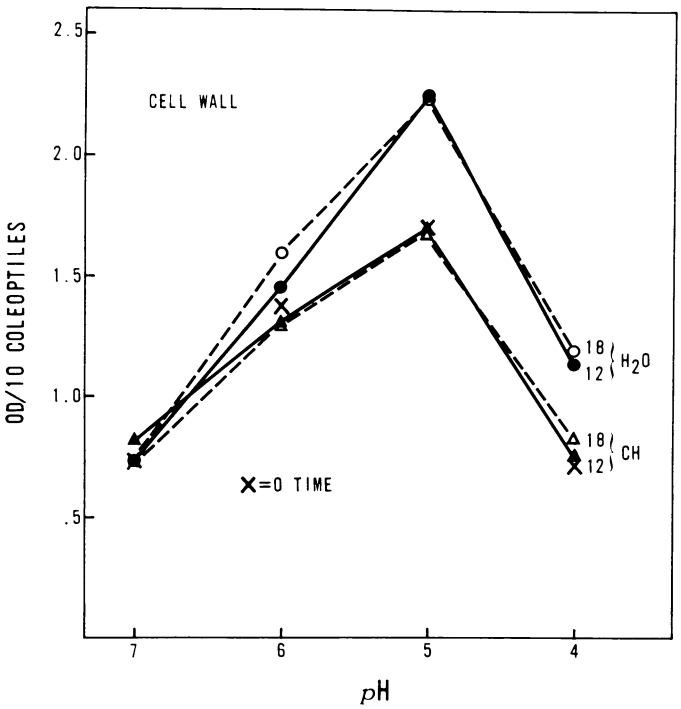

FIG. 2. Stability of cell wall-bound β-galactosidase. Coleoptile segments were preincubated for 0, 12, or 18 hr in the dark at 22 C in distilled H_2O or a 10 μg/ml cycloheximide (CH) solution, after which cell walls were quantitatively prepared and assayed for β-galactosidase activity.

Table II. *Effect of IAA on β-Galactosidase Activity in Intact Coleoptile Segments*

Segments were pretreated for 60 min at 30 C in 1.5 ml of 1 mM phosphate-citrate buffer (pH 6.0) with or without 0.1 mM IAA, then assayed by the addition of 0.5 ml of 40 mM *p*-nitrophenyl-β-D-galactopyranoside. The data are an average of duplicate or triplicate samples.

Experiment	Enzyme Activity	
	− IAA	+ IAA
	absorbance units/10 coleoptiles	
1	0.345	0.440
2	0.345	0.485
3	0.335	0.425
4	0.360	0.470
5	0.345	0.565
6	0.345	0.450
7	0.350	0.450
8	0.365	0.505
Averages	0.349	0.474

known properties of galactosidases from other sources (29). Furthermore, 0.1 N HCl treatment irreversibly inactivates this enzyme which correlates with the effect of 0.1 N HCl on extension growth of frozen-thawed *Avena* coleoptiles (Rayle and Cleland, unpublished data).

During the course of this study, it came to our attention that cycloheximide treatment of excised coleoptiles for up to 22 hr did not appreciably reduce the capacity of *Avena* coleoptile segments to extend in response to H^+ (R. Cleland, personal communication). Is the cell wall-bound β-galactosidase similarly insensitive to cycloheximide treatment? To pursue this question, excised coleoptile segments were incubated in the presence or absence of 5 μg/ml cycloheximide for 12 or 18 hr, then cell wall fractions were prepared quantitatively and assayed for β-galactosidase activity. As seen in Figure 2, cycloheximide treatment did reduce slightly the measurable β-galactosidase activity compared to H_2O-treated coleoptiles, particularly when assayed at the lower pH regions. However, compared to the enzyme activity of cell walls isolated from fresh coleoptiles (no preincubation), cycloheximide treatment was without effect. We interpret these results to mean that cell wall-bound β-galactosidase is completely stable for up to 18 hr, but cycloheximide blocks the increase in enzyme activity that results when segments are incubated on H_2O. Such an increase may reflect deposition of new enzyme into the wall with time, a process which is likely to be susceptible to cycloheximide action.

Since low pH increases the activity of *Avena* β-galactosidase, then it follows that IAA treatment of nonpeeled or peeled coleoptiles, by virtue of enhancing H^+ release from the cytoplasm to the cell wall, should also stimulate β-galactosidase activity in the wall. We have observed IAA-induced enhancements of β-galactosidase activity for both nonpeeled and peeled coleoptile segments, and Table II shows such results for a number of experiments done with nonpeeled segments. The average promotion after 60 min of preincubation in 0.1 mM IAA was 36% over control. Slight IAA enhancements (10–15%) were obtained after 30 min of hormone pretreatment, but the data border on statistical insignificance due primarily to a limited number of trials. Similar effects were found for peeled segments, but owing to the difficulty in removing all or even a constant amount of the cuticle-epidermis layer during the peeling procedure, the replicate samples assayed for available β-galactosidase were quite variable. No IAA effect on this enzyme activity could be observed when isolated cell walls were incubated with the hormone (unpublished data). The point to be made, though, is that since IAA does cause H^+ extrusion from these cells (4, 22) and β-galactosidase in the wall has an acidic pH optimum, at least part of the hormonal enhancement of this enzyme activity undoubtedly results from the presumed secondary messenger of IAA action: hydrogen ions.

Abscisic acid (25) and cycloheximide (3) are known to rapidly inhibit IAA-induced elongation of *Avena* coleoptiles and may do so by rapidly inhibiting IAA-induced H^+ secretion (4, 22). These inhibitors were also completely effective in blocking an IAA-induced increase in β-galactosidase activity (Table III).

If the mechanism by which IAA enhances cell elongation involves the release of H^+ which initiate wall loosening, then it follows that the IAA-induced extension of peeled segments (*i.e.* where there is no cuticular barrier to H^+ and buffer flux) at pH values of 6.0 or above should be subject to inhibition by high buffer strengths. This is indeed the case and has been recently reported (7). When a similar experiment was run to test the effect of buffer strength on the IAA-induced increase in β-galactosidase activity, essentially analogous results were obtained (Fig. 3). For the sake of better precision in replicate samples, nonpeeled coleoptile segments were used for this experiment instead of peeled segments (the latter responded similarly but with greater variability). Since all the measureable β-galactosidase activity is present at or near the cut ends of the segments (Fig. 1b), the nonpeeled segments should behave as peeled segments with respect to the enzyme activity assayed, although total activity would be less. These results taken with the above mentioned growth data suggest that the action of IAA involves the release of some bufferable substance (H^+)

that can cause an enhancement of β-galactosidase activity as well as elongation.

Another indication that IAA causes the release of H^+ which subsequently increase wall-associated β-galactosidase activity arises from the results of an interaction experiment between IAA and pH. Figure 4 illustrates that as the pH of the preincubation and assay medium was lowered, the relative IAA effect was diminished. This result fits the criteria established by Lockhart (15), whereby two regulators either act at the same site, or that one regulator acts to increase the level of the other. Since IAA acts to increase the level of H^+ in the wall (4, 22), then the decreased IAA effect at lower pH values probably results from a comparatively smaller contribution of H^+ elicited by IAA relative to the external medium at pH 4.0. We may also deduce from this experiment a crude estimation of the magnitude of the IAA effect on H^+ secretion, by comparing the level of β-galactosidase activity in the presence of IAA at pH 6.0 to the pH value that yields a similar level of enzyme activity without IAA. Under the conditions of this experiment, we calculate that treatment of nonpeeled seg-

Table III. *Inhibition of the IAA-enhanced β-Galactosidase Activity by Abscisic Acid and Cycloheximide in Intact Coleoptile Segments*

Ten segments/flask were pretreated for 30 min at 30 C in 1.3 ml of 2 mM phosphate-citrate buffer (pH 6.0) with or without 10 μg/ml cycloheximide or 20 μM abscisic acid. After 30 min, 0.2 ml of 1 mM IAA or H_2O was added to the flasks and allowed to incubate for 60 min, followed by the addition of 0.5 ml of 40 mM *p*-nitrophenyl-β-D-galactopyranoside for 30 min. The data are an average of triplicate flasks.

Inhibitor	± IAA	Enzyme Activity
		Absorbance units/10 coleoptiles
None	−	0.260
	+	0.365
Abscisic acid	−	0.266
	+	0.268
Cycloheximide	−	0.263
	+	0.267

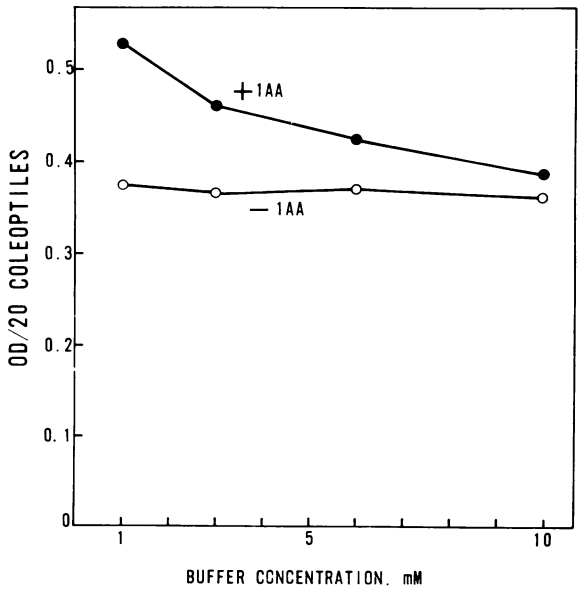

FIG. 3. Effect of increasing buffer strength on IAA-induced enhancement of β-galactosidase activity. Nonpeeled segments (10 mm) were incubated 60 min at 30 C with or without 0.1 mM IAA at the indicated concentration of K-phosphate buffer, pH 6.0, then assayed for β-galactosidase activity.

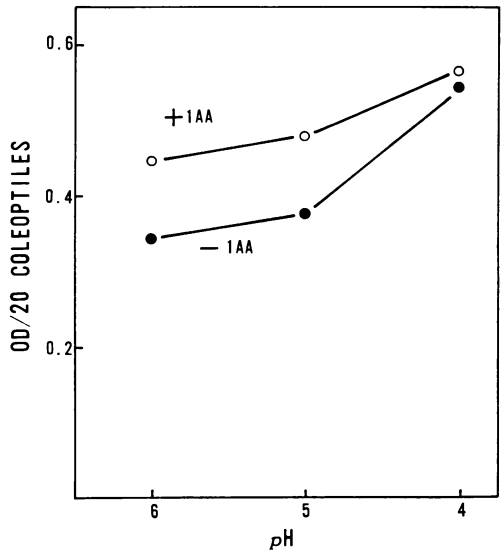

FIG. 4. Interaction of IAA and pH on β-galactosidase activity of intact coleoptile segments. Nonpeeled segments were incubated for 60 min at 30 C with or without 0.1 mM IAA in 1 mM phosphate-citrate buffer (variable pH), then assayed for β-galactosidase activity.

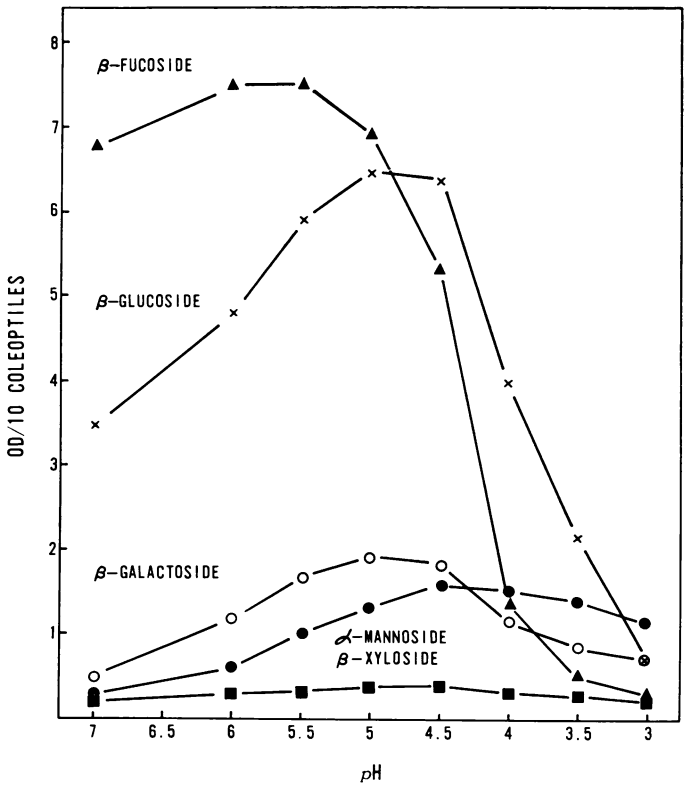

FIG. 5. Comparative pH activity profiles for *Avena* coleoptile cell wall-bound glycosidases. Cell walls were prepared and assayed as described in "Materials and Methods." Final substrate and buffer concentrations were 10 mM. All the substrates listed (except *o*-nitrophenyl-β-L-xyloside) were *p*-nitrophenyl-glycoside derivatives. Inactive substrates yielded less than 0.3 *A* units/10 coleoptile cell walls at any pH between 7 and 3.5, and include *p*-nitrophenyl-α-fucoside, -α-galactoside, -α-glucoside, and -β-mannoside.

ments with 0.1 mM IAA can reduce the pH of the cut-end region from pH 6.0 (1 mM buffer) to about 4.6 after 60 to 90 min of hormone treatment. β-Galactosidase is not the only glycosidase present in *Avena* cell walls. There have been previous reports of the occurrence of glycosidases in *Avena* cell walls (10, 12, 16) as well as other plant sources (1, 5, 6, 14, 20, 21, 27). No reports to our knowledge, however, have attributed any significance to the acidic pH optima of some of the glycosidases relative to cell extension. At least four glycosidase activities (β-galactosidase, β-glucosidase, α-mannosidase, and β-xylosidase) present in *Avena* cell walls exhibit pH optima between 4.5 and 5.0 (Fig. 5). The pH activity curves for β-galactosidase and β-glucosidase in peeled coleoptiles and isolated cell walls, however, show a rapid decline in activity below pH 4.5 (Figs. 1a and 5), whereas H^+-induced extension of peeled coleoptiles is equally effective between pH 3 to 5, at least when initial rate measurements are taken (22). This makes it difficult to assign a causal relationship to any one of these particular enzymes in pH 3-induced extension, but this does not preclude such a role for glycosidases in general. The α-mannosidase exhibits a broad pH optimum between 3 and 5 (Fig. 5), as does a β-arabinosidase recently isolated from *Cajanus* seeds (6). The correlation between any of the acid-activated *Avena* glycosidases and auxin-induced cell elongation, however, must await measurements of actual cell wall acidity generated *in vivo* by IAA treatment.

The reduction in cell wall pH resulting from H^+ secretion occurs rapidly enough to be considered one of the primary effects of IAA in causing cell elongation (4, 22). Can we say the same for localized bond-splitting in cell walls? Labavitch and Ray (13) have presented preliminary evidence that IAA causes the release of a xyloglucan polymer from etiolated pea stem sections as rapidly as it promotes elongation of the segments. Whether or not this is a direct antecedent to IAA-enhanced cell elongation remains to be seen. The only direct evidence that glycosidases may be involved in cell elongation is the work of Masuda and co-workers (19, 28) who demonstrated that a fungal β-1,3-glucanase substantially promotes cell elongation of *Avena* coleoptiles. Others, however, have encountered difficulty in confirming these observations (2, 21, 26).

Another difficulty in assessing the role of the *Avena* glycosidases in cell elongation has to do with the putative wall-loosening step. If wall loosening involves the breakage of internal polysaccharide bonds within the wall matrix enzymatically, the wall-loosening enzyme should presumably exhibit an endo-splitting action, such as the β-glucanase used by Masuda and others (19, 28). Since *p*-nitrophenyl-glycosides were used in this study, our data only reflect the activities of exo-glycosidases. Therefore, although the results show a strong correlation between the activity of (exo)-β-galactosidase and auxin- or H^+-induced cell extension, a direct role for this and other exoglycosidases in the cell wall loosening step seems improbable. They may, however, contribute indirectly to wall loosening by further degrading polysaccharide chains that have suffered an initial internal cleavage by an endo-glycosidase or act to incorporate new wall material into the cell wall via their known transferase activity (29).

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