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Auxin-Enhanced Glucan Autohydrolysis in Maize Coleoptile Cell Walls[1]

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ABSTRACT

Cell walls isolated from auxin-pretreated maize (*Zea mays* L.) coleoptile segments were assayed to disclose evidence for the existence of enhanced autolysis. To improve the sensitivity of the measurements and to facilitate kinetic analysis, isolated cell walls were consolidated within a small column, and the autolysis rate was directly determined from the sugar content of the effluent. This protocol revealed that the maximum rate of autohydrolysis of walls prepared from segments occurs within the first 2 hours and a steady decline commences almost immediately. Walls from indoleacetic acid pretreated segments (0.5–4 hours) released sugar at a higher rate initially (110–125% of controls) and the enhanced rate of autolysis continued for 6 to 8 hours, but then it became equivalent to that of the controls. Pretreatment of the segments at acidic pH had no effect on the measurable rates of autolysis. The (1→3),(1→4)-β-D-glucan content of the walls and the extractable glucanase activities support the hypothesis that temporal enhancement of autohydrolysis is a function of auxin on enzyme activity. The progressive decline in autolysis during prolonged incubations is consistent with the decrease in the quantity of the β-D-glucan in the wall. The relationship between glucan content and autolysis rate is supported by the observation that while glucose pretreatment of segments had only a small effect on initial autolysis rates, the presence of the sugar during pretreatment served to extend the interval over which higher rates of autolysis could be sustained. The results demonstrate that autolysis is related to auxin-induced wall metabolism in maize coleoptiles.

Cell extension is regulated by the capacity of the wall to accommodate the acquisition of water by the protoplast, and the enhancement of elongation in response to auxin is a function of specific adjustments in the physical properties of the cell wall (1, 19, 27). Auxin-induced changes in the cell wall have been correlated with quantitative and qualitative changes in certain matrix polysaccharides in the Poaceae (2, 11, 16, 21, 24) and certain dicot cell walls (15, 23).

Of all the quantitative changes in cell wall composition mediated by auxin, the effect on β-D-glucans of cereal coleoptile appears to be the most significant. Loescher and Nevins (16, 17) reported that IAA treatment of *Avena* coleoptile segments caused a decrease in the noncellulosic glucan content of the cell walls. This IAA response has been confirmed in other cereals including maize, rice, and barley (6, 25, 32). In addition, notable changes in cell wall glucan occur in coleoptiles during the development of intact maize seedlings (18). The net result is a transient increase followed by a decline in the β-D-glucan content. Such changes are correlated with growth of the coleoptile. These observations also suggest a relationship between glucan metabolism and auxin responses.

Isolated maize coleoptile cell walls exhibit autohydrolytic activity (autolysis). In the process of autolysis, noncellulosic β-D-glucans are degraded ultimately to glucose (7–10) by an endoglucanase and an exoglucanase associated with the cell wall (5, 9). Based on these observations, we hypothesize that auxin regulates the action of one or both of these enzymes. To provide support for this contention, *in vitro* studies were conducted to establish the effect of auxin on autolysis. While Huber and Nevins (9) reported little difference in the extent of autolysis that could be attributed to auxin, others have shown that auxin-enhanced activity of glycanases occurs in cereal coleoptiles (12, 14, 20, 28). A reconciliation between the various observations is needed.

In the present study, we employed a sensitive method to measure the initial changes in the rate of autolysis to disclose putative transients that might persist as a consequence of auxin pretreatment. We then proposed to identify changes in enzyme activity that might be expected to account for differences in the autolytic rate.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays* L., hybrid B37 × Mo17) caryopses were imbibed for 24 h in running water at 27°C and sown on wet vermiculite in plastic trays. The seedlings were maintained under red light for 24 h and then transferred to darkness for 48 h. Coleoptiles were excised at the basal node and held in distilled water. After rinsing, the coleoptiles were recut and the central 5 mm section was bathed in a 10 mM K-phosphate buffer (pH 6.5) in the presence or absence of 30 μM IAA and/or 30 mM glucose for 0.5 to 6 h at 26°C. In some experiments, the effect of pretreatment at acidic pH on growth was measured. In acid growth experiments, coleoptile surfaces were abraded with moist carborundum powder (320 mesh) before cutting the initial 10 mm sections. Abraded segments were subsequently recut into 5 mm sections and treated with 10 mM K-citrate phosphate buffer (pH 4.0 and 6.5).

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Autolysis

Coleoptile segments treated with IAA and/or glucose were washed with distilled water and were immediately frozen at −70°C. For each autolysis treatment, 20 segments were used. The frozen segments were homogenized in 10 mL of 50 mM NaCl at 0°C and the insoluble wall material was transferred to a compact chromatography column (8 × 40 mm, Bio-Rad). The walls retained within the column were washed extensively with 50 mM NaCl (0°C), acetone (−20°C), and again with 50 mM NaCl. The column was equilibrated with buffer (20 mM Na-citrate phosphate, pH 5.6) at 0°C. After equilibration, the remaining buffer was removed by suction. Autolytic reactions were allowed to proceed for 2 h intervals at 37°C in the presence of 1 mL of the buffer. Material solubilized during a 2 h interval was collected by suction, and 1 mL of fresh reaction buffer was immediately introduced to the column for the next assay interval. Cell walls heated to 100°C for 5 min served as controls.

Assay of Exo- and EndoGlucanases

Cell wall proteins were extracted with 3 M LiCl as reported (5, 8, 10). Extractions were performed *in situ* by adding 0.5 mL of 3 M LiCl directly to the cell walls (2 mg fresh weight) contained within the column. The walls were maintained with the LiCl solution for 3 h at 4°C. The LiCl extracts were collected by imposing a suction. Salts in the extract were removed with the aid of a NAP-5 column (9 × 28 mm, Pharmacia) after the column had been equilibrated with 20 mM K-phosphate buffer (pH 6.5). Desalted extracts were applied to a hydroxylapatite column (9 × 20 mm, Bio-Rad) that had been equilibrated with the same buffer. The retained proteins were eluted with 0.3 M K-phosphate buffer (pH 6.5). The buffer was exchanged for 20 mM K-citrate phosphate (pH 5.6) with the aid of a NAP-5 column. The activities of exo- and endoglucanases were assayed employing β-D-glucan (0.5% w/v), purified from *Avena* bran, in the presence and absence of 100 μM $HgCl_2$. (See ref. 8 for details). Activities, expressed as glucose equivalents, were determined from the rate of release of reducing groups (26).

Determination of the Noncellulosic Glucan Content

Noncellulosic glucan content in cell walls was determined by the method of Luttenegger and Nevins (18). After the completion of an autolysis experiment, the walls contained within the column were heated to 100°C for 5 min in a water bath. Inactivated cell walls were treated with Porcine pancreatic-amylase (Sigma, Type I-A) for 12 h at 37°C and then washed with distilled water. The cell walls were then incubated for 24 h at 37°C with a β-D-glucanase purified from Novo Ban 120 L (Novo Industri A/S, Copenhagen, Denmark) (13). Total sugars released were determined by the phenol sulfuric acid method (3) and expressed as glucose equivalents.

All autolytic and enzymatic assay incubations were conducted in the presence of 0.05% NaN_3.

RESULTS

Influence of IAA Pretreatment of Coleoptiles on Wall Autolysis

Autolysis of the β-D-glucan of maize cell walls is well documented. Those studies show that in the course of the reaction the initial products are more highly polymerized, but with time the appearance of glucose predominates. The sequence in the production of products is consistent with the action of two glucanases, one capable of releasing oligomers and one with the ability to degrade the oligomers to glucose. The regulation of autolysis and the enzymes responsible is less clear. The rate of release of products appears most rapid initially but as a function of incubation time it gradually declines. Since autohydrolysis is predominantly directed toward the degradation of β-D-glucan (7), the decline in the rate during extended incubations may be a consequence of the depletion of the available substrate (Fig. 1). We observe that the reaction proceeds until nearly all the endogenous substrate is hydrolyzed. However, one would expect that the available substrate should have little influence in restricting the initial reaction rates. Although both treatments are shown in Figure 1, differences in the overall expression of autolysis in response to IAA pretreatment of sections are difficult to discern simply by examination of the appearance of accumulated sugars in the bathing medium with conventional procedures (7) (Fig. 1).

To more precisely resolve the auxin effect on rates of the initial autolytic sequence, small quantities of cell wall material (about 1 mg fresh weight) were taken directly from a prescribed number of segments pretreated with and without

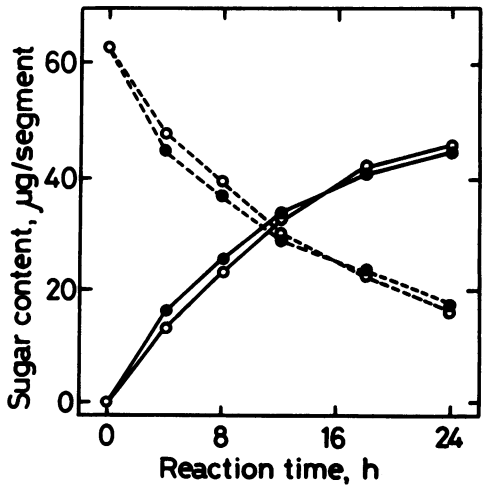

Figure 1. Autolysis and residual β-D-glucan content of maize cell walls. Cell walls were prepared from maize coleoptile segments (5 mm in length) that had been pretreated with or without 30 μM IAA for 2 h. The cell walls prepared from the segments were allowed to undergo autolysis in 10 mL of 20 mM K-citrate phosphate buffer (pH 5.4) at 37°C for 24 h. The sugars released during the course of autolysis (solid lines) were determined by the phenol-sulfuric acid method (13). The residual noncellulosic β-D-glucan content in the cell walls (broken lines) was estimated by an enzymatic method (18). ●, IAA; ○, control.

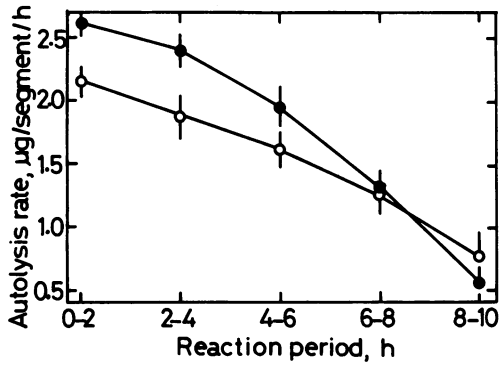

Figure 2. Effect of IAA on the initial rates of autolysis. Cell walls, prepared from 20 coleoptile segments treated with or without IAA for 2 h, were allowed to autohydrolyze while confined to a column. The incubation buffer was replaced at 2 h intervals. The reaction mixtures were collected by suction, and the released sugars were determined by the phenol-sulfuric acid method. Data are presented as combined means, ± SE, of three separate experiments. ●, IAA; ○, control.

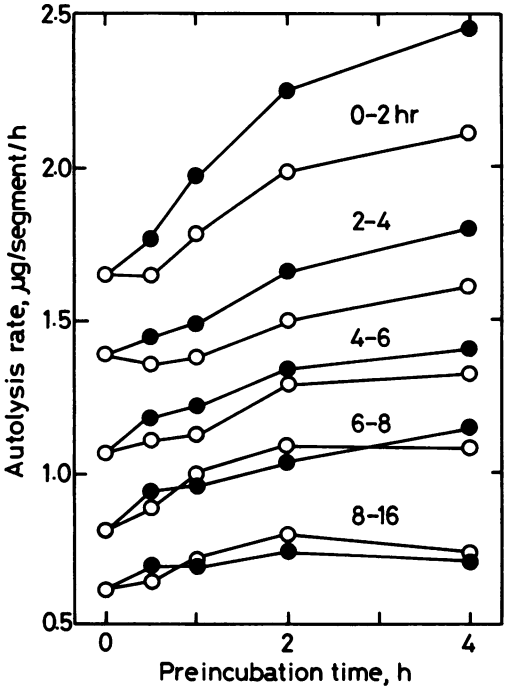

Figure 3. Effect of IAA preincubation time on subsequent autolysis rates. Coleoptile segments (5 mm in length) were treated with 30 µM IAA for 0.5 to 4 h, and the cell walls were allowed to undergo autolysis in a column as described for Figure 2. ●, IAA; ○, control. The times given in hours refer to specific autolysis intervals.

auxin for 2 h. In such studies, the overall rates of autolysis exhibited maximum levels within the first 2 h. Thereafter, the autolytic rates progressively decreased. However, during the initial phase, IAA pretreatment of segments consistently showed elevated autolysis rates (Fig. 2). IAA enhancement of autolysis approaches 125% those of the controls. After approximately 4 h, the rates declined and began to converge. By 8 h, the enhancement induced by auxin was no longer apparent.

When auxin pretreatment of sections was extended to 4 h, the rates of autolysis were similar to those stimulated by 2 h pretreatments (Fig. 3). The auxin effect was diminished when the preincubation time was less than 2 h. Nevertheless, trends suggest an auxin effect when auxin pretreatment was provided for 30 min. Regardless of the length of auxin exposure during a pretreatment, the effect on autolysis persisted over the first 8 h.

Effect of Hydrogen Ions on Autolysis

Protons may stimulate certain early auxin responses (1, 4). Acidic buffers (pH 4.0) also induce cell elongation, although the effect is generally of less magnitude than that observed when sections are exposed to auxin (Table I). We observed, however, that pretreatment of sections in acidic buffers promoted no measurable effect on subsequent autolysis of isolated cell walls (Table I). To evaluate the direct effect of pH on autolysis, we incubated sections with and without auxin for 4 h, prepared walls from the segments, and measured autolysis in the presence of buffers in a pH range from 4 to 7. Autolysis exhibited the same pH responsiveness regardless of the autolysis interval measured, and the optimum for activity remained at approximately 5.5 (Fig. 4). When data are expressed on a per section basis, it is clear that the auxin enhancement is maintained in a manner consistent with that observed in previous experiments, *i.e.* an enhancement is greatest during the first 2 h and that difference is ameliorated after about 6 h. In general, the profiles of the pH responses are similar and the character of the curves was maintained even after the auxin effect was no longer expressed (after 6–8 h). The results suggest that pH influences the rate of autolysis by affecting the pH environment for the enzymes and that this response is different from that induced by auxin pretreatment.

Table I. *Effect of pH on Cell Extension and the Rates of Cell Wall Autolysis of Maize Coleoptile Segments*

The coleoptile segments (5 mm), were abraded with carborundum and treated with 10 mM K-citrate-phosphate buffer (pH 4.0 and 6.5) for 2 h at 25°C. Cell walls were prepared from the segments and autolysis was measured as described in Figure 2. Means ± SE.

Treatment	Cell Elongation[a]	Autolysis Rate[b]
	mm	*µg/segment/h*
pH 6.5	0.05 ± 0.01	2.53 ± 0.14
pH 4.0	0.36 ± 0.03	2.44 ± 0.08

[a] n = 20 segments. [b] n = mean of three experiments.

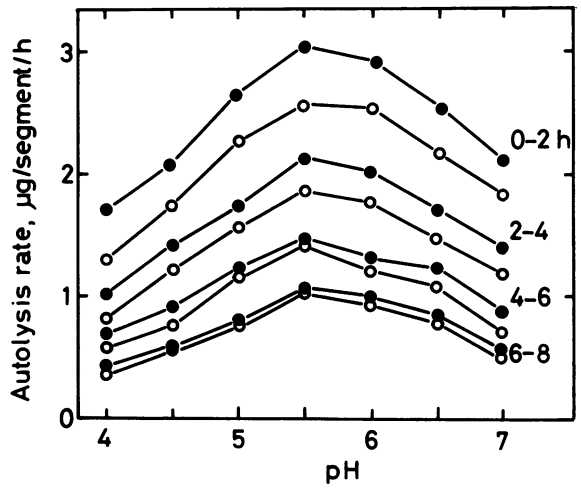

Figure 4. pH responses of autolysis as a function of incubation time. Cell walls were prepared from 20 coleoptile segments pretreated with and without auxin for 4 h. Autolysis was allowed to proceed in 1 mL of Na-citrate-phosphate buffer (20 mM, pH 4–7). Other conditions are given in Figure 2. ●, IAA; ○, control. The times given in hours refer to specific autolysis intervals.

Effect of Glucose Pretreatment on Subsequent Autolysis

The presence of glucose or sucrose in the incubation medium during exposure to auxin is known to diminish the extent of hormone mediated *in vivo* changes in the cell wall β-D-glucan content (16). Two possibilities may account for this glucose effect. Perhaps the presence of glucose serves to regulate polymer hydrolysis directly, or alternatively the presence of sugars might provide a supply of substrate available for continued wall synthesis to replace regions deleted by hydrolysis. No direct effect of sugars on autolysis or autolytic enzymes has been found. Therefore, it would appear that an abundant supply of sugar precursors contributes to maintaining the wall composition at a steady state. We observed that including glucose in the pretreatment medium had little effect on the rate of subsequent autohydrolysis, but more importantly, glucose served to sustain autolysis for a longer period of time (Fig. 5). We also observed that the longer the sections were pretreated with glucose, the longer the interval that the enhancement in the autolysis rate was sustained (data not shown).

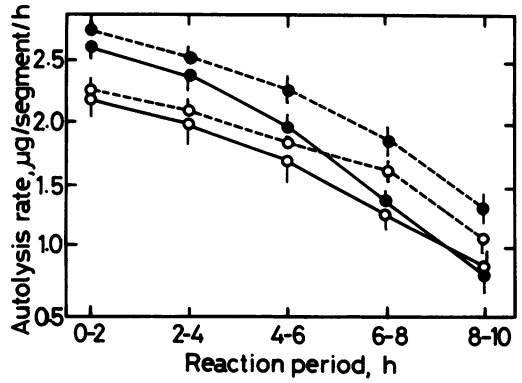

Figure 5. Effect of pretreatment of segments with glocuse on the rate of cell wall autolysis. Coleoptile segments were treated with 30 mM glucose and/or 30 µM IAA for 4 h. Cell walls prepared from these segments were allowed to undergo autolysis as described for Figure 2. ●, IAA; ○, control. The solid lines refer to pretreatment in the absence of glucose and the broken lines refer to pretreatment in the presence of glucose. Data are presented as combined means, ± SE, of three separate experiments.

Effect of Auxin on Activities of Glucanases Extracted from Maize Coleoptile Cell Walls

The presence of auxin as a pretreatment for segments stimulated the recovery of the extractable glucanase activities up to 300% (Table II). The data are therefore consistent with the hypothesis that auxin-enhanced autolysis is mediated by increased activities of cell wall glucanases. However, it is noted that after 8 h autolysis, the recovery of enzyme activity was lower. At that point, the activity of the enzyme assayed in the absence of $HgCl_2$ was one-half that of the initial levels, but a more significant reduction in activity was observed by reactions conducted in the presence of $HgCl_2$. The results suggest that the activity of the endoglucanase declines to a greater extent than the exoglucanase (9). Nevertheless, some IAA enhancement of glucanases persists even after 8 h.

DISCUSSION

Treatment of cereal coleoptile segments with auxin results in a decline in the cell wall noncellulosic glucan content. A correspondence between these events and cell extension has been established (16, 17, 24, 25, 32). In addition, these correlations have been extended to relate glucan metabolism to the expression of cell wall mechanical properties (19, 22). Nevertheless, the mechanisms that account for auxin-mediated glucan metabolism remain unresolved. Huber and Nevins (9) sought to examine auxin effects on cell wall autolysis in maize coleoptile segments, but found no evidence for hormonal regulation of the process during extended autolysis intervals. Similar results were obtained when we re-

Table II. *Effect of Auxin on Glucanase Activities Extracted from Maize Coleoptile Cell Walls*

Cell walls from 40 coleoptile segments pretreated with and without exogenous auxin were sampled immediately after the pretreatment period and again after 8 h of autolysis. Enzymes were extracted with 3 M LiCl (see text for details). The LiCl extracts were desalted and incubated with 0.1 mg·mL^{-1} *Avena* glucan in the presence and absence of 100 µM $HgCl_2$ (see ref. 9). The assay was based on the release of reducing sugars. Means are shown ± SE.

Treatment	−$HgCl_2$[a]		+$HgCl_2$[b]	
	Initial	After 8 h[c]	Initial	After 8 h
	µg glucose reducing groups released·h·40 segments^{-1}			
Control	1.54 ± 0.11	1.16 ± 0.05	0.49 ± 0.03	0.08 ± 0.01
IAA	5.01 ± 0.61	2.56 ± 0.29	0.90 ± 0.06	0.15 ± 0.02

[a] Three hour incubation for enzyme assay. [b] Sixteen hour incubation for enzyme assay. [c] Enzyme activities were assayed after 8 h of autolysis.

examined those experiments (Fig. 1). However, upon a more critical analysis of initial autolysis, we found that auxin consistently enhances the rates within the first few hours (Fig. 2). The results allow for a further consideration of a role for auxin in the regulation of wall glucan metabolism in cereal coleoptiles.

Auxin enhancement of initial rates of autolysis was evident with a 30 min pretreatment (Fig. 3), suggesting that it may be one of the early responses of the tissue to the hormone. Thirty minutes was the shortest pretreatment interval employed in the current studies. Since wall acidification has been cited as a mediator of initial auxin effects (2, 4) in cell elongation, an evaluation of its role in autolysis was sought. The function of lowered pH may be directed toward nonenzymatic adjustment in wall components, but it is more likely to be considered as a mechanism for affecting the activity of resident enzymes (2, 11, 12). We found that acidic pH pretreatment of sections has no effect on subsequent autolytic rates. Moreover, an evaluation of autolysis in response to pH revealed no evidence that protons complemented the enhancement expressed by auxin pretreatment (Fig. 4). One would not expect that acidic buffers used would directly modify noncellulosic glucans (17, 24). The effect of auxin pretreatment on subsequent autolysis appears independent of cell wall acidification.

Auxin-enhanced autolysis is most likely the result of the hormone on the level of enzyme activity responsible for glucan metabolism (21, 30, 31). We observed that cell wall proteins prepared from IAA treated maize coleoptile segments exhibited enhanced glucanase activities (Table II). Both enzymes responsible for autolysis were affected: the exoglucanase and the endoglucanase. In other words, glucan degradation by enzymes extracted from IAA pretreated walls was higher when assays were run both in the presence and absence of $HgCl_2$. One may conclude that among the rapid effects of gene expression in response to auxin (29) may be an enhanced synthesis of glucanases. However, the enzyme complement stimulated by IAA is imposed on a relatively abundant level of constitutive glucanases. Other gene expression products that modify the activity of the enzymes must also be considered.

Two other observations relate to the interpretation of a role for auxin in the enhancement of autolysis. The extent of the stimulation of autolysis is a function of the time of preincubation with auxin. Hence, what we observe is an enhancement of autolytic rate in response to auxin that continues to increase beyond the time required for auxin to initiate the maximum stimulation in elongation rate. The continued stimulation of autolysis may indicate that small changes in enzyme levels may be responsible for affecting the capacity of the wall to expand. Clearly the growth of these sections is sustained at a constant rate well beyond the time required for the maximum stimulation of autolysis. Also of interest is the observation that after several hours of autolysis, the enhanced rate dissipates (Fig. 2). In other words, the rates stimulated by auxin pretreatment eventually decline to converge with those of the controls. The decrease in the rate of autolysis may be related partially to the decline in the glucanase activities. We observed that the endoglucanase activity decreased to about 15% of the enhanced level after an 8 h autolysis interval (Table II). Since substrate levels were not limiting in the *in vitro* assays, one must consider that other factors are responsible for this decline in activity. In extended incubations, the depressed autolytic rates are, however, probably a result of the depletion of substrate. We conclude that the effect of IAA on the autolysis rate may be a function of enzyme activities. However, since these activities did remain higher even when the stimulation of autolysis was no longer apparent, other factors may be involved. A consideration must be made of the consequences of events that modify the wall structure, especially in the absence of the biosynthesis of new polymers. Collectively, these changes may impart a limitation to enzyme accessibility to the substrate.

Auxin is reported to have little or no measurable effect on the noncellulosic β-D-glucan content in walls of *Avena* coleoptile segments *in vivo* within the first hour (16). This is most likely the result of continued glucan synthesis utilizing endogenous precursors to replace those components removed. In the presence of a supply of exogenous glucose or sucrose, the net decline in glucan content is suppressed. We found that the presence of glucose did not directly inhibit *in vitro* autolysis, suggesting that glucose does not govern autolysis by an allosteric mechanism. And, while glucose in the preincubation medium had some effect on the initial autolytic rates, the predominant response of the pretreatment was to sustain higher autolytic rates for a more extended interval. In these experiments, exogenous glucose supplied to sections would be expected to enrich the cell wall in β-D-glucan content for the initial stages of autolysis and thereby allow the process to occur at higher rates for a longer interval. Convergence of the auxin treatment with the controls was effectively delayed when these pretreatments were used. The results may point to a more important role for the maintenance of the concentration of glucan in the walls as a regulator of expressed rates of autolysis. The results offer additional approaches in resolving the relationship.

The auxin-enhanced hydrolysis of cell wall β-D-glucans may serve several roles in cell extension. The modification of the polymer may directly contribute to changes in the mechanical properties of the cell wall to accommodate expansion, and the hydrolysis of the polymer may supply substrates for other metabolic events closely linked to cell extension. For example, glucose released from the wall matrix may be used for osmotic adjustment, for respiratory metabolism, and for reincorporation to sustain wall synthesis. The fact that auxin pretreatment enhances *in vitro* β-D-glucan autolysis and establishes higher glucanase activities offers opportunities to further explore the role of these events in auxin-induced elongation.

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