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Role of Cell-Wall-Degrading Enzymes in Cell-Wall Loosening in Oat Coleoptiles

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Summary. Effects of auxin (indole-3-acetic acid), fungal β-1,3-glucanase and pectin methylesterase on expansion and on cell wall extensibility, measured by the extensometer technique, of oat coleoptile segments were studied. Pretreatment with these substances for less than 30 min promoted tissue expansion remarkably. Under osmotic stress by 0.25 M mannitol, which prevented uptake of water by the cells, auxin increased DE but not DP in 30- and 60-min incubations. β-1,3-Glucanase or β-1,3-glucanase plus pectin methylesterase also increased only DE under the same conditions. A role of cell-wall-degrading enzymes in initiating cell expansion is therefore suggested.

Introduction

A number of hypotheses concerning the mechanism by which auxin induces rapid cell elongation have been proposed [1, 16, 20]. The majority of these hypotheses have dealt with auxin-induced cell wall loosening since auxin was early shown to make the cell wall more extensible [11]. It has recently been established that nucleic acid and protein metabolism is involved in the action of auxin to induce cell elongation and cell-wall loosening [2, 4, 8, 14, 20, 23—26, 30, 31]. It is thus probable that auxin-induced cell elongation and cell-wall loosening result from the synthesis of a certain enzyme (or certain enzymes).

We have already reported that a fungal β-1,3-glucanase isolated from cultures of *Sclerotinia libertiana* induced rapid elongation of excised oat coleoptile segments [21, 27, 39]. The enzyme was also shown to increase cell-wall extensibility, as measured by a stretching method [27]. A contribution of cell-wall-degrading enzymes in cell elongation has recently been suggested and, in fact, activities of some enzymes, particularly of glucanase and hemicellulases, have been found in a cell-wall fraction [13, 17] and to be increased by auxin [15, 37]. It is thus possible that auxin induces cell elongation primarily by regulating the activity of certain cell-wall-degrading enzymes which affect mechanical properties of the cell wall.

For measuring cell-wall properties, three kinds of techniques have long been employed, i.e., bending, plasmolytic and stretching methods. Each of these, however, involves some disadvantages in technical convenience or in evaluating the results obtained [6]. The Instron extensometer technique which was developed by Olson et al. [33] and by

CLELAND [5] overcame some of the disadvantages. CLELAND [5, 6] has recently scrutinized the Instron technique and refined its methodology.

Some critical problems in measuring cell wall extensibility should be considered when we are to know the significance of auxin-induced changes in the mechanical properties of the cell wall leading to cell elongation. Changes in wall properties of cells elongating some hours in the presence of auxin may be a reflection of growth potential for further elongation and the result of the elongation which has taken place. For this reason, it is important to employ a system where cells are treated with auxin but without elongation, so that we may be able to see the direct effect of auxin on cell-wall properties. CLELAND [5] has shown that auxin increases the plastic extensibility of oat-coleoptile cell walls even under osmotic stress by 0.25 M mannitol, although the increase was quite small. He paid more attention to plastic (DP) than to elastic (DE) extensibility since the former is increased by auxin more conspicuously than the latter [5, 6].

According to CLELAND'S kinetic study, DP increases following the administration of auxin, reaches a maximum within 1 to 2 hours, and then remains constant during further incubation. A statistically significant increase in DP due to auxin was reported to be observed 20 min after auxin administration. The growth rate reached a maximum and constant level in 10 to 15 min following auxin addition [24, 36]. It is thus doubtful that DP properly represents the growth potential.

The present study was designed to obtain information on the detailed relationship between auxin-induced elongation and changes in cell wall extensibility, and on the nature of the biochemical changes in the cell wall initiating changes in its mechanical properties.

Material and Methods

Plant Material. Oat (*Avena sativa* L., cv. Victory) seedlings were irradiated with weak red light for 1 day after germination and grown in the dark at 25° thereafter [24, 39]. The apical 2 mm of coleoptiles 30 ± 3 mm long were decapitated, and 2 hours later a 4- or 15-mm segment was excised from the upper region of the coleoptile, and the enclosed first leaf removed.

Growth Test. 20 segments, 4 mm in length, were floated on the test solution and the length was traced using a low power ($\times 20$) microscope under dim green light at intervals of 30 to 180 min.

Expansion experiments were also performed to separate in time the action of auxin on the coleoptile cells from the cellular expansion by water uptake [7]. Coleoptile segments were subjected to 4 successive treatments [7]: (1) The segments were pooled in 10 ml of 0.25 M mannitol solution which made them slightly shorter than the original 4 mm. (2) After 30 min the segments were randomized and a group of 20 segments was transferred to another 10 ml of mannitol solution containing or not containing 10 mg/l indole-3-acetic acid (IAA) or enzymes (β-1,3-glucanase or pectin methylesterase; abbreviated as β-G and PM, respectively) and incubated

for 10 to 120 min at 25°. (3) The treatment was then followed by a transition treatment (washing) with plain 0.25 M mannitol solution for 10 min. (4) The segments were finally allowed to expand in 10 ml water and the length was determined after 30, 60 and 120 min.

Force-Extension Analysis. Segments, 15 mm in length, variously treated, were killed by immersion for 5 min in boiling methanol [5, 6, 33]. The segments were then treated with 200 µg/ml Pronase for 18 hours to remove protein. They were returned to methanol and stored.

Force-extension analyses were performed using a "Tensilon" model UTM-II tensile-tester of Toyo Sokki Co. It can be operated in practically the same way as an "Instron" extensometer [33]. The distance between the clamps was 5 mm and the rate of lowering of the bottom clamp was 2 mm/min. The load-extension curve was automatically recorded (chart speed: 20 cm/min, see Fig. 1). The segments were finally removed from the clamps, dried and weighed for calculating the mass per unit length (M/L) [5, 6].

The slope of the load-extension curve increased until a force of 15 to 20 g was reached; the force then increased in a linear manner, as extension proceeded. Since the force equivalent to the longitudinal stress exerted by turgor pressure was supposed to be between 20 and 40 g [6, 38], the extension was discontinued when the load reached 40 g, the clamps were returned to their original positions and the segment was extended again. As clearly shown by CLELAND, the first and the second load-extension curves were conspicuouly different from each other. Following his formulae, the compliance describing the elastic extensibility (DE) in the 30 to 40 g force range could be obtained from the slope of the second curve at a force of 35 g, after obtaining the total compliance of total extensibility (DT) from the slope of the first curve. Finally, the compliance of plastic extensibility (DP) could be obtained by subtracting DE from DT. In summary, calculation procedures were as follows:

$$\mathrm{DT} = \frac{\Delta L/L}{F} \cdot \frac{M}{L} \cdot \frac{1}{\varrho}\ (\mathrm{cm^2/dyne}),$$

$$\mathrm{DE} = \frac{\Delta L'/L'}{F} \cdot \frac{M}{L} \cdot \frac{1}{\varrho},$$

$$\mathrm{DP} = DT - DE,$$

where L = the initial length at which a line tangent to the curve at 35 g crossed the abscissa, and ΔL = extension from the first load-extension curve; L' and $\Delta L'$ were those from the second curve; ϱ = density, assumed to be unity; $\Delta L/F$ and $\Delta L'/F$ = the co-tangent of the lines and $\frac{M}{L} \cdot \frac{1}{\varrho}$ a substitution for F/A (F: force across the segment; A: the cross-sectional area of the wall). Fig. 1 illustrates the treatment for obtaining DT and DE, and thus DP.

We also calculated "extensibility", following the method of OLSON et al. [33] from the slope of the straight portion (co-tangent) of first and second load-extension curves. However, growth capacity of oat-coleoptile segments seems, in general, to be correlated better with DE and DP values than with "extensibility". The values presented in Experimental Results are averages and standard errors of 20 or 22 measurements, using different coleoptiles.

Enzymes. Fungal β-1,3-glucanase (β-G) and pectin methylesterase (PM) isolated from a culture of *Sclerotinia libertiana* [9, 32] were kindly supplied by Drs. Y. SATOMURA and S. OI, Laboratory of Microbiological Chemistry of our University. The enzymes were extracted from the culture and fractionated successively

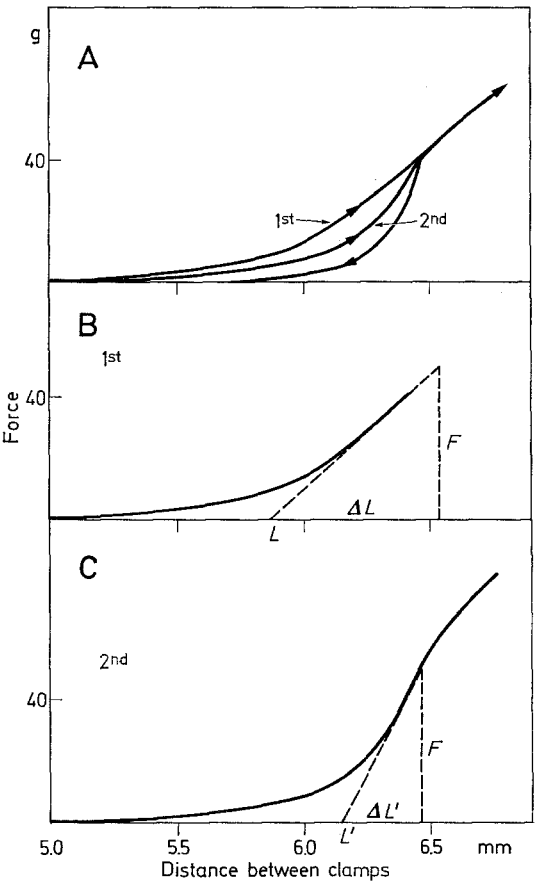

Fig. 1. Typical load-extension curves (A) and method for determining plastic and elastic extensibilities (B and C). (A) A segment was extended to 40 g load (1^{st}), then returned to the original position (back arrow), and extended again (2^{nd}). The segment was extended at 2 mm/min. (B) Method for determining total extensibility (DT). Slope of the curve was determined at 35-g load. (C) Method for determining elastic extensibility (DE). Slope of the curve was determined at 35 g load

on columns of Duolite-A2, Amberlite CG-50 and CM-cellulose (PM: pH 3.6; β-G: pH 4.2). Fractionated enzyme preparations were then precipitated by ammonium sulfate and dialyzed. The β-G was contaminated only with traces of cellulase and PM activities [9, 27, 39]. The enzyme activity of a β-G solution was referred to as 1 unit/ml when 1 mg glucose was produced from 10 mg Sclerotan (*Sclerotinia* glucan) incubated in the enzyme solution (0.02 M acetate buffer, pH 4.5) for 20 min at 40° [9]. The activity of a PM solution was defined as 1 unit/ml when 2×10^{-5} moles of free carboxyl residues were liberated from 5 ml of 2% citrus-pectin solution incubated in the enzyme solution (in 1.5 M NaCl) for 60 min at 30° [32]. Enzyme activities per mg protein were 40.0 units for β-G and 47.3 units for PM [9, 32].

Experimental Results

a) Expansion Experiments. Reproducibility of coleoptile expansion pretreated with or without auxin under osmotic stress was quite satisfactory, as can be seen in Table 1 and as also reported by CLELAND and BONNER [7]. The standard errors indicate that generally a difference of 0.08 mm or more between L values was statistically significant at the 5% level. The expansion (ΔL in 120-min expansion) was entirely proportional to the length of the period of IAA pretreatment, in confirmation of CLELAND and BONNER [7; see Fig. 5].

Table 1. *Residual effect of auxin on expansion of oat coleoptile segments*

Expt. No.	Final length (L), mm		L (+)—L (−)
	−IAA	+ IAA	
1	4.28 ± 0.035	4.51 ± 0.032	0.23
2	4.28 ± 0.042	4.52 ± 0.046	0.24
3	4.27 ± 0.025	4.51 ± 0.029	0.24
4	4.28 ± 0.029	4.53 ± 0.035	0.25
5	4.29 ± 0.032	4.52 ± 0.031	0.23
Average	4.28	4.52	0.24

Pretreatment with or without 10 mg/l IAA in 0.25 M mannitol for 60 min. Expansion period: 120 min; initial length: 3.96 mm.

We have previously reported that a fungal β-G induced a rapid elongation of oat and barley coleoptiles and pea internode segments [27, 37, 39]. We tried to see the effect of this cell-wall-degrading enzyme on the elongation of oat segments under osmotic stress. In addition, the effect of PM was observed as a role of PM in inducing cell-wall loosening was extensively discussed some years ago [cf. 3, 10, 34], and auxin was reported to increase the activity of this enzyme in pea-stem segments [40].

In a preliminary experiment reported previously [27] the optimal concentration range of β-G was found to be 0.1 to 0.3 units/ml. Fig. 2 shows the interaction between 0.1 unit/ml β-G and varying concentration of PM in inducing coleoptile elongation. PM alone showed only little effect on elongation, but greatly enhanced the effect of β-G in a certain range of its concentration. A time-course experiment showed (Fig. 3) that β-G induced rapid elongation, almost as rapid as 10 mg/l IAA in the first hour of incubation. The promotion of elongation by the enzyme then became small. Addition of PM to β-G enhanced the elongation.

Fig. 4 shows a similar effect of these enzymes on the expansion of coleoptile segments which were pre-incubated for 30 min in 0.25 M mannitol solution containing or not containing these enzymes or IAA.

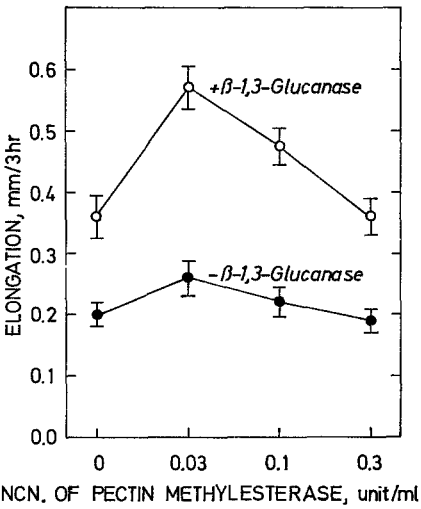

Fig. 2. Growth response of excised segments of oat coleoptiles to varying concentrations of fungal pectin methylesterase (PM) in the presence or absence of 0.1 unit/ml fungal β-1,3-glucanase (β-G). Vertical bars represent standard errors

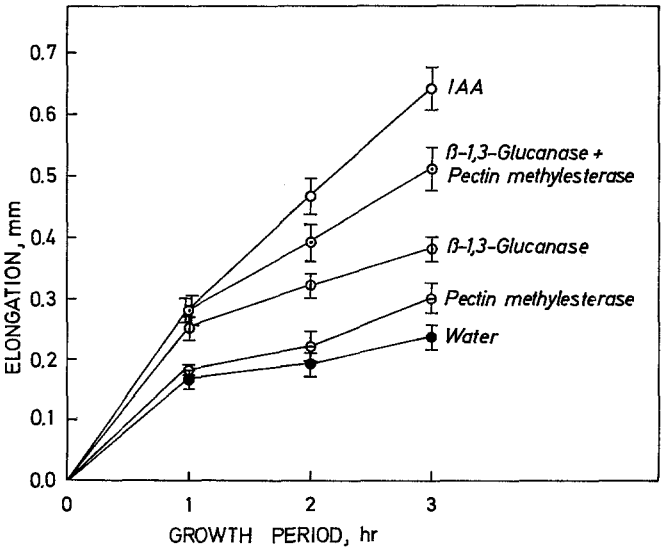

Fig. 3. Time course of elongation of excised segments of oat coleoptiles in 0.1 unit/ml fungal β-G and/or 0.05 units/ml fungal PM. IAA: 10 mg/l. Vertical bars represent standard errors

The pretreatment with β-G plus PM seemed to promote the expansion more than that with IAA. The relationship between expansion and the length of pretreatment with these enzymes and auxin is given in Fig. 5.

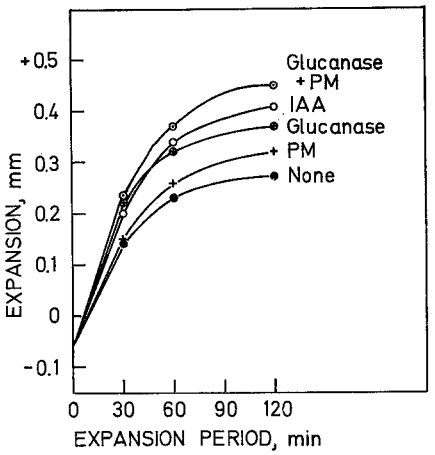

Fig. 4. Time course of expansion in water of excised segments of oat coleoptiles. Segments were pretreated in 0.25 M mannitol containing or not containing 10 mg/l IAA, 0.1 unit/ml β-G and/or 0.05 units/ml PM for 30 min and transferred to water after a transition period of 10 min

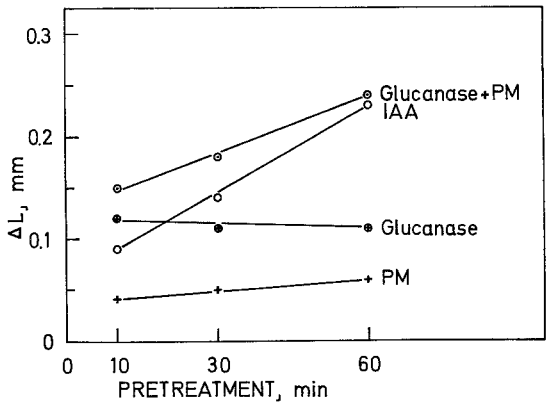

Fig. 5. Relationship between expansion in water and length of pretreatment with 10 mg/l IAA, 0.1 unit/ml β-G and/or 0.05 units/ml PM in 0.25 M mannitol. Expansion period: 120 min

The effect of β-G was large after a 10-min pretreatment, but the effect was not increased by increasing the time of pretreatment, whereas this was the case with β-G plus PM and with IAA. The addition of PM, however, greatly enhanced the effect of β-G even in a short incubation period, and the longer the pretreatment the greater the enhancement. The administration of β-G and PM together resulted in a pattern of expansion of coleoptile segments similar to that with IAA, i.e., the longer the pretreatment the greater the residual effect (expansion in water).

b) Force-Extension Analysis. The effect of auxin on the cell-wall extensibility of turgescent cells was compared with that of non-turgescent cells under osmotic stress by 0.25 M mannitol. Values of DE and DP were plotted against the length of the incubation period in the presence and absence of auxin (Figs. 6 and 7). Differences in the values between no mannitol and 0.25 M mannitol may have resulted chiefly from

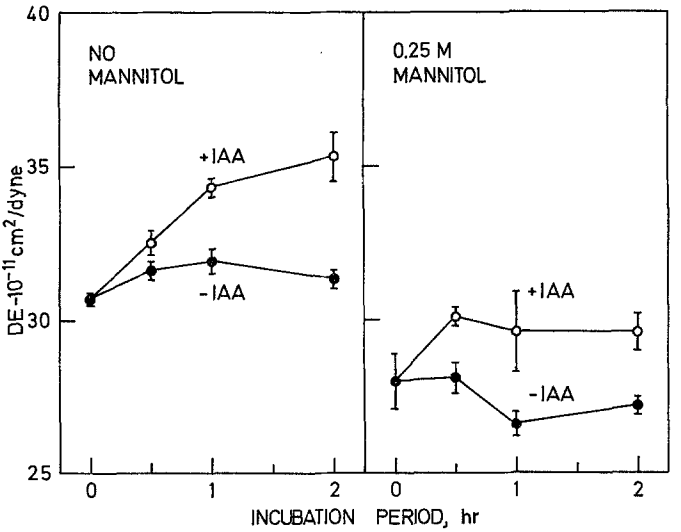

Fig. 6. Time course of change in DE caused by IAA in the presence or absence of 0.25 M mannitol. Segments were incubated in test solutions for 0 to 2 hours, killed and DE determined. Values are averages of 20 or 22 separate measurements. Vertical bars represent standard errors

the elongation which had taken place during the incubation period. IAA significantly increased DP in 1 hour in the absence of mannitol, but not in the presence of mannitol. IAA, however, increased DE in as little as 30 min under the osmotic stress. In addition, the effect of IAA on DE under osmotic stress did not increase even when coleoptile segments were treated with IAA for 2 hours. Under no osmotic stress, DP and DE increased gradually as the time of IAA treatment increased. An effect of IAA on DP was noticed also in the presence of mannitol, after 120 min of treatment.

Solutions of β-G, PM, and β-G plus PM containing 0.25 M mannitol caused an increase in DP after 60 min but no apparent effect after 30 min incubation (Fig. 8). DE, on the other hand, was significantly increased by β-G or β-G plus PM more in 30 min than 60 min incubation. DE again seemed to go in parallel with the degree of expansion to follow (cf. Figs. 4 and 5). The situation is clear when we see the effect of β-G

on DE and DP. DE was increased by β-G in 30 min incubation and the effect rather reversed if incubation was further extended. Thus, in the case of β-G, DE parallels the expansion to follow. In the case of IAA and

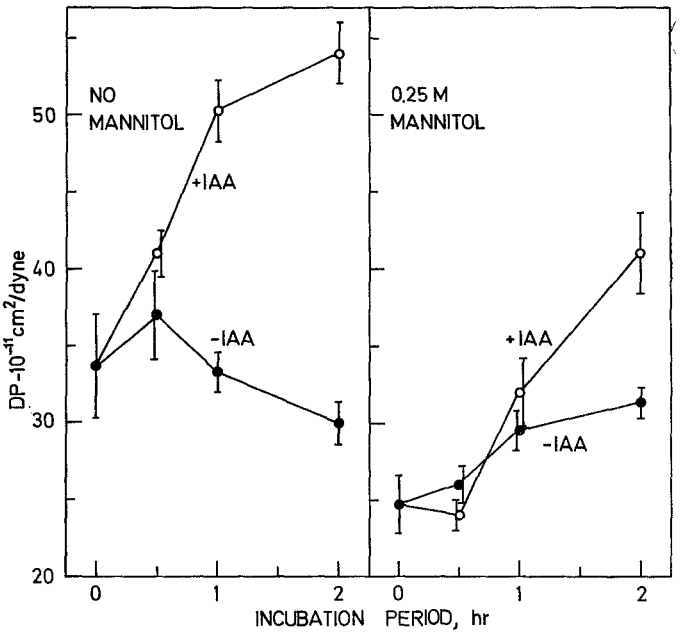

Fig. 7. Time course of change in DP caused by IAA in the presence or absence of 0.25 M mannitol. Segments were incubated in test solutions for 0 to 2 hours, killed and DP determined. Values are averages of 20 or 22 separate measurements. Vertical bars represent standard errors

Table 2. *Effect of heated β-1,3-glucanase on expansion and cell wall extensibility*

Treatment	Expansion in 90 min (mm)	Extensibility, $\times 10^{-11}$ cm²/dyne	
		DE	DP
None	4.27 ± 0.0018[a]	28.3 ± 0.23	30.2 ± 1.26
β-G, 0.1 unit/ml (heated)	4.27 ± 0.014[a]	28.1 ± 0.25	29.6 ± 1.28
β-G, 0.1 unit/ml (not heated)	4.42 ± 0.028[a]	30.4 ± 0.17	28.6 ± 1.12

Coleoptile segments, 4 mm or 13 mm in length, were pooled in 10 ml of 0.25 M mannitol solution. After 30 min segments were randomized and a group of 20 segments were transferred to another 10 ml of 0.25 M mannitol solution containing or not containing 0.1 unit/ml β-G either heated for 10 min at 70° or not heated, and incubated for 30 min at 25°. After washing, 4-mm segments were allowed to expand in water for 90 min. 13-mm segments were killed and DE and DP determined. Values are averages of 20 separate measurements with standard errors.

[a] Mean length before expansion: 3.98 ± 0.016 mm.

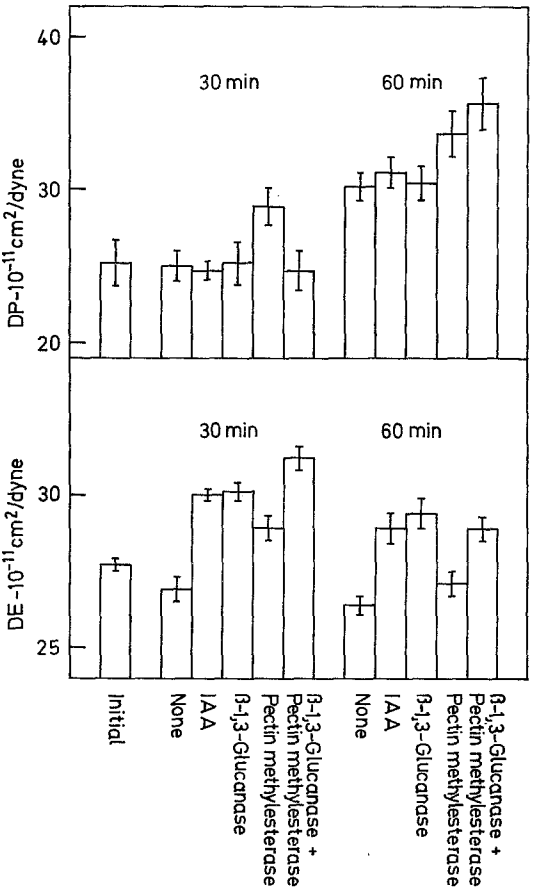

Fig. 8. Effect of auxin and cell-wall-degrading enzymes on DP and DE. Segments were incubated in 0.25 M mannitol containing or not containing 10 mg/l IAA, 0.1 unit/ml β-G and/or 0.05 units/ml PM for 0, 30 and 60 min, killed, and DP and DE determined. Values are averages of 20 or 22 separate measurements. Vertical bars represent standard errors

β-G plus PM, too, as with β-G alone, DE initially (in 30 min) increased, whereas DP tended to increase only following further incubation (60 min). β-G heated for 10 min at 70° had no effect either on expansion or on DE or DP in a 30-min incubation (Table 2).

Discussion

Using the Instron technique CLELAND, observing that auxin greatly increases DP, concluded that auxin increased the cell-wall extensibility by acting on the strain-hardening function rather than by acting on some

form of viscoelastic flow. There is, however, a difficulty in correlating the growth potential with DP, as CLELAND [5, 6] himself pointed out. After administration of auxin, growth rate reaches a maximum within 10 to 15 min [24, 27, 36] whereas DP does so after 90 to 120 min. Although we have not yet done a detailed kinetic study, we find that DE reaches a maximum level already in 30 min under osmotic stress (in fact, even in 15 min, although data are not given here), with the level remaining roughly constant during a further 1- to 2-hour incubation period (Fig. 6). The growth potential increased by IAA thus is primarily reflected by the increase in DE; in other words, the bigger the growth potential the bigger the value of DE. This need not necessarily mean that the increase in DP due to auxin has nothing to do with cell expansion. In fact, even under osmotic stress DP increases 2 hours after the administration of IAA (Fig. 7). However, DP does not seem primarily to regulate the growth potential since IAA brought about no significant increase in DP in 30 to 60 min incubation under osmotic stress. It may thus be possible that the direct effect of auxin on cell-wall extensibility is primarily represented by the increase in DE.

As is well established, the growth rate is regulated by at least three factors: the osmotic potential, the permeability of cells to water, and the cell-wall extensibility. Concerning the change in cell-wall properties which are supposed primarily to regulate the growth potential we should consider not only cell-wall loosening but also certain kinds of cell-wall synthesis, such as a tensile synthesis of cell wall, as suggested by RAY [35]. Our main interest is in the kinds of biochemical and biophysical changes in cell walls which primarily initiate growth. Studies on metabolic turnover in oat cell walls using ^{14}C showed that the label in a hemicellulose-rich fraction decreased accompanying increased growth [39]. This result suggests a possible relation of metabolic turnover of hemicellulosic polysaccharide to cell-wall extension. Some investigators have also reported breakdown or metabolic turnover of cell-wall constituents during growth [12, 18, 28]. We have proposed the hypothesis that an enzymatic degradation of hemicellulosic polysaccharides, the major component of the cell-wall matrix, causes an increase in cell-wall extensibility, leading to cell elongation [22, 39]. Apparent increase in cell wall extensibility, particularly in DE, due to β-G or β-G plus PM supports the hypothesis, implying that cell-wall degrading-enzymes such as glucanases or PM play a crucial role in initiating elongation. It is thus most important to clarify the nature and mode of action of cell-wall-degrading enzymes acting on wall constituents such as hemicellulosic polysaccharides. Although we have reported that cellulase did not play an important role in inducing cell extension [39], this enzyme may be considered to be important in some cases [19].

One aspect appears to be puzzling: Treatment with PM alone resulted in a rapid increase in DP even though the enzyme gave only little promotion of elongation or expansion. The elongation of coleoptile tissue, as a whole, is a sum of the elongations of individual cells, and could be limited by cell-adhesiveness, which is supposed to be determined by the middle lamellae which consist mainly of pectic substances. Reduction in adhesiveness alone, possibly caused by such an enzyme as PM, would not contribute to the elongation of whole tissue. Once individual cells start to elongate, for example due to the action of β-G, reduction of cell-to-cell adhesiveness may make elongation of the tissue easier. DP values obtained by the extensometer technique may not only indicate the extensibility of individual cell walls, but may be affected also by the adhesiveness between cells. Auxin thus might cause a reduction of adhesiveness between cells, as well as a loosening of the walls of the individual cells.

Since there is evidence that nucleic acid and protein metabolism is involved in auxin action (see Introduction), cell-wall-degrading enzymes may be synthesized following the addition of auxin. In considering the primary action of auxin we should of course not pay attention only to the synthesis and mode of action of cell-wall-degrading enzymes. Evidence by MORRÈ [29] suggests that direct involvement of certain enzymes which limit cell expansion, other than enzymes required for cell-wall loosening, is adequate to support growth. Thus, the effect of auxin on both cell-wall degradation and re-synthesis (extensile synthesis) should be considered.

In conclusion, we may say that the growth potential is regulated both by elastic (DE) and plastic (DP) extensibilities of cell walls. An increase in DE is required for the initiation of cell expansion and an increase in DP is needed, possibly, to maintain a high, constant growth rate. The former could be increased, at least partly, by the degradation of hemicellulosic components, and the latter perhaps by extensile new cell-wall synthesis and reduction of adhesiveness between cells.

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