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FORCE EXTENSION ANALYSIS OF AVENA COLEOPTILE CELL WALLS

By

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With 3 Figures in the Text

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Summary

A method is described for measuring the cell wall mechanical properties of *Avena* coleoptiles in the absence of turgor stress or influences of a living protoplast. Force-extension curves obtained with a constant-rate-of-extension instrument and standard fiber-testing techniques demonstrate the permanence of cell wall loosening effects of prior indoleacetic acid (IAA) treatment of living tissue and provide evidence that these changes involve interactions between cell wall polymers. By this method various chemical and enzymatic modifications of cell walls can be evaluated in terms of altered mechanical properties. Thus, it was possible to remove over 97% of the cell nitrogen (including some hydroxyproline-containing protein) by hot methanol followed by enzymatic treatment and not change the extensibility properties of the tissue. In contrast, coleoptile mechanical properties were markedly influenced by chemical acetylation procedures or cellulase treatment.

Introduction

The increased tissue extensibility associated with indole-3-acetic acid-(IAA) induced growth or growth potential has led to the widely held concept that IAA regulates cell expansion through a reduction of wall pressure (BONNER, 1961; BURSTRÖM, 1961; HEYN, 1933, 1940; PRESTON and HEPTON, 1960). Relaxation of cell wall bonding under cytoplasmic control is implied but the manner of wall loosening and the chemical nature of contributing cell wall constituents remains obscure (WILSON, 1964).

We were interested in what components of the wall contribute to coleoptile mechanical properties, the extent and magnitude of the chemical changes induced in coleoptile structure by IAA, and the possibility of determining mechanically what specific components were altered by IAA treatment. Load-extension curves were obtained on coleoptiles, both before and after specific modifications of IAA-treated and untreated sections, by standard fiber-testing procedures. Such curves have been

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widely used to illustrate differences in resistance to stretching between fibers, to show the effects of chemical modification on the mechanical properties of fibers, and to help establish a physical picture of fiber structure (MORTON and HEARLE, 1962).

Material and Methods

Oat seedlings (*Avena sativa*, L. var. Siegeshafer) were grown in vermiculite in stainless steel trays from seeds that had been presoaked 4 hours in distilled water with continuous aeration. The seedlings were grown in low-intensity red light at 25^0 C and 90% relative humidity until they measured 2.5 to 3.5 cm from tip to coleoptilar node. After harvesting, the top 3 mm apical section was discarded and the leaf removed.

Coleoptile sections were floated on 0.0025 *M* solutions of potassium maleate buffer, pH 4.5, with and without 10^{-5} *M* IAA for 1 to 8 hours. These coleoptiles followed the time course for elongation summarized by BONNER (1961). Unless otherwise specified the coleoptiles used were treated 6 to 8 hours.

Coleoptiles were killed by a 5-minute treatment in boiling methanol. Sections were then stored in cold methanol and rehydrated in water prior to measurement. Most of the chemical and enzymatic treatments were performed on methanol-treated coleoptiles rehydrated in water.

Dry sections were prepared by placing methanol-treated coleoptiles between layers of Miracloth, a porous fabric, for several hours to prevent curling of the coleoptiles as they dried.

Dry weights of samples were obtained after 12—24 hours at 60^0 C. Nitrogen was determined by microkjeldahl analysis.

Coleoptiles were treated with a solution of 0.25 mg per ml Pronase, a protease from *Streptomyces griseus* (California Corporation for Biochemical Research) in 0.03 *M* sodium phosphate buffer, pH 7.4, containing 5% ethanol (NOMOTO, NARAHASHI and MURAKAMI, 1960). Cellulase (Worthington Biochemical Corporation) was used in aqueous solution containing 1.0 mg/ml.

Soluble or extractable protein was obtained by grinding 100 to 150 coleoptiles in 15-ml Kontes series B all-glass homogenizers in 0.01 *M* tris-0.25 *M* sucrose buffer, pH 7.2. Pellets were extracted 5 to 8 times, the extracts combined, protein precipitated with 15% trichloroacetic acid, and washed several times with ethanol followed by ethanol-ether. The soluble protein and the cell walls remaining after extraction were hydrolyzed and assayed for proline and hydroxyproline (OLSON, 1964).

Load-extension curves were obtained with a constant-rate-of-extension instrument, an Instron Universal Testing Instrument (Instron Engineering Corporation, Canton, Massachusetts). Coleoptile sections were fastened vertically between two clamps preset 0.65 cm apart. With the upper clamp fixed, the lower clamp was then traversed down at a constant rate of 0.127 cm/min, increasing the load on the coleoptile. Load was measured electrically by a resistance strain-gauge connected to the upper clamp. The extension of the coleoptile was controlled by an error-operated servo-system.

Coleoptiles were held by Standard No. 2 grips with leather squares 2 to 4 mm thick fitted on the metal faces of the grips by a 2-way adhesive tape, Scotch Brand No. 400. Coleoptiles were firmly clamped hand-tight in the grips with the rough side of the leather gripping the specimen. No slippage of the samples was observed under these conditions, as verified in experiments in which the gauge-length was varied from 0.2 to 1.2 cm. The same differences between IAA- and non-IAA-treated coleoptiles were observed over this entire gauge-length range. Contributions of slippage at the

grips toward the extension would be constant and, as the gauge-length was shortened, the percent extension would have greatly increased if slippage were a serious problem.

The plot of the load across a coleoptile in grams against increase in length in cm (a force-extension chart) is shown in Fig. 1. The numbers used to compare different treatments were obtained from the slope of the first straight portion of the force-extension curves, the co-tangent, BE, and expressed in cm/g of applied load. The larger this number, the greater the extensibility of the coleoptile for the same load. Under these conditions, the average length of coleoptile was estimated by back extrapolation of the extension curve to be 0.70 cm. Extensibilities recorded represent averages of 5 to 10 determinations.

Results

The extensibilities for non-IAA-treated and IAA-treated coleoptiles are 1.0×10^{-3} cm/g and 2.3×10^{-3} cm/g. The load for a 10% extension

Table 1. *Break-load and extensibility of coleoptiles*[1]

Treatment	Break-load g		Extensibility cm × 10³/g	
	Control	IAA	Control	IAA
None (fresh tissue)	60	40	1.0	2.0
Dried tissue[1]	145	130	0.23	0.33
Methanol, boil 5 min	70	50	1.0	2.3
Ethanol, boil 5 min	75	50	1.0	2.3
Water, boil 5 min	70	50	1.0	2.3
Acetone, boil 5 min	60	50	1.5	2.3
Ethanol, then benzene, boil 5 min in each	50	50	1.3	2.3
Methanol-chloroform (1—2), boil 5 min	65	50	1.3	2.5
Ethyl acetate, boil 5 min	70	55	1.3	2.0
Freeze on dry ice	65	45	1.3	2.3
All of the following boiled 5 min in methanol, stored in methanol, and rinsed in water just prior to treatment:				
0.1 *M* NaOH 23° C 5 min	60	40	1.5	3.8
0.1 *M* HCl 23° C 8 hours	60	50	1.8	2.3
0.1 *M* HCl, boil 5 min	35	35	2.3	4.1
Acetic acid, boil 5 min	70	55	1.3	2.3
Acetic anhydride, boil 5 min	90	80	0.5	1.0
Pectinase	65	55	1.0	2.3
Pronase	65	50	1.0	2.3
"Cellulase"	10	10	2.5	5.1

[1] All measurements except those for dried tissue were made at a load extension rate of 0.127 cm per minute. For dried coleoptiles a load extension rate of 0.0254 cm per minute was used. Mean variation in extensibility was $\pm 0.2 \times 10^{-3}$ cm/g. For dry tissue it was $\pm 0.03 \times 10^{-3}$ cm/g. Mean variation in break-load was $\pm$ 15 g.

is 73 g for non-IAA-treated and 32 g for IAA-treated coleoptiles, the percent extension for a load of 50 g is 7 and 16, respectively, and the percent extension at the break points is 10 and 16, respectively. (Figs. 1 and 2.)

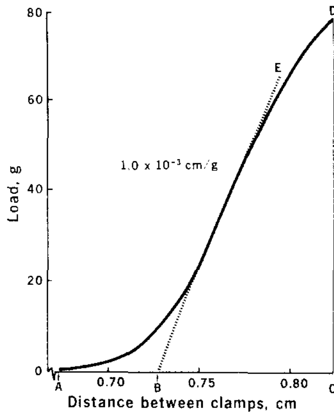

Fig. 1

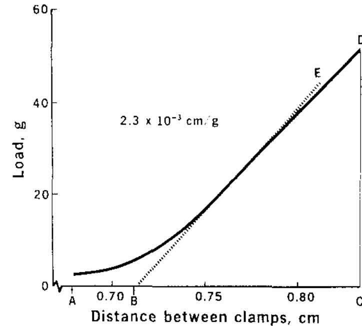

Fig. 2

Fig. 1. Force-extension curve of *Avena* coleoptile section obtained on an Instron Universal Testing Instrument. Non IAA-treated section boiled 5 minutes in methanol and rehydrated in water prior to measuring. Extension 0.127 cm per minute. Zero distance between the clamps is not shown on the chart. Point A represents the length of the coleoptile after it has just been clamped and while it still has some slack or "crimp" in it. BE is drawn tangent to the initial straight part of the curve and extrapolated to zero load. The distance to B represents an approximate length of the straightened uncrimped coleoptile. Point D is the breaking point of the coleoptile, line CD is the breaking load, and line BC is the extension at the point of rupture, the breaking extension

Fig. 2. Same as in Fig. 1 except section had been incubated in IAA 7 hours prior to methanol treatment and measurement

A change in the extensibility of coleoptiles is observed after a one-hour treatment in IAA, e.g., 1.6×10^{-3} cm/g for treated sections compared to 1.0×10^{-3} cm/g for controls. The change due to IAA approached a maximum value in 2 to 3 hours.

Break-loads, the load at which the coleoptile separates at a point of maximum weakness, are given in Table 1. Breaks result from weak areas in the coleoptile and 40

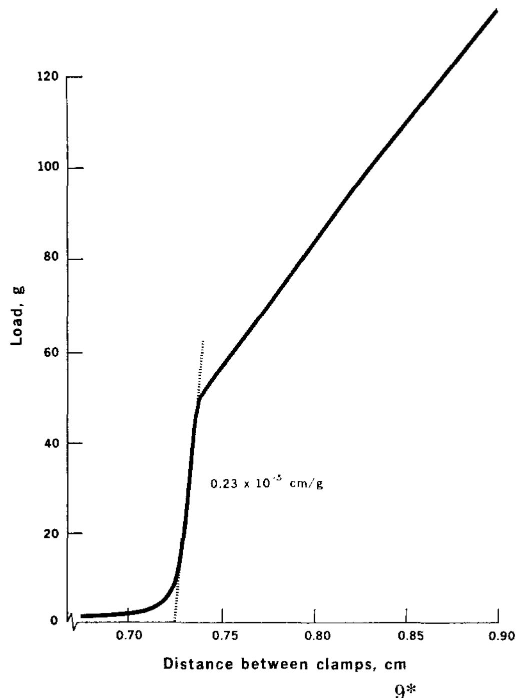

Fig. 3. Force-extension curve of non-IAA-treated dry section. For conditions see footnote, Table 1

to 60% of the breaks occurred at or near the grips. The break-load for IAA-treated coleoptiles was always less than that for the corresponding non-IAA-treated coleoptiles.

The extensibility of turgid coleoptiles, compared with those for coleoptiles treated with hot water, hot methanol, or a variety of organic solvent combinations, or freezing shows that these treatments have little effect on the force-extension measurements (Table 1). In addition to removal of water, over 50% of the dry weight of non-IAA-treated coleoptiles was lost by boiling them in methanol (Table 2).

Table 2. *Coleoptile dry weight and nitrogen content*

Treatment	Dry weight micrograms/cm	Nitrogen micrograms/cm
None	630	28
Boiled 5 min in methanol	240	12
Boiled 5 min in methanol, treated with buffer 17 hrs	220	10
Boiled 5 min in methanol, treated with buffered Pronase 17 hrs	170	0.5

Drying methanol-treated coleoptiles increased the break-strength and decreased the extensibility (Table 1). In addition, force-extension curves of dry coleoptiles showed a definite yield point or load at which the extensibility increased. This is not observed in force-extension curves of hydrated coleoptiles (Figs. 1 and 3). Increased extensibility and decreased break-strength due to IAA treatment were still apparent in measurements of dry coleoptiles (Table 1).

Treatment of coleoptile sections with 0.1 *M* NaOH for 5 minutes at room temperature, boiling 0.1 *M* HCl for 5 minutes, or cellulase for 17 hours increased extensibility (Table 1).

Incubation of sections for 17 hours at 37° C with Pronase resulted in no change in either the extensibility or break-strength of the sections. Pronase removes most of the nitrogen in the coleoptile as well as considerable other material as shown from the nitrogen and dry-weight loss (Table 2). The presence of a hydroxyproline-rich cell-wall protein in coleoptile cell walls is shown by the data in Table 3. Sixty to seventy percent of the total hydroxyproline in the cell is in the cell wall and is resistant to attack by the methanol followed by Pronase treatment.

The mechanical properties of methanol extracted coleoptiles can be markedly altered by both chemical and enzymatic treatments as shown

in the data of Table 4. Treatment of coleoptiles with cellulase weakens wall structure, whereas treatment of coleoptiles with acetic anhydride results in cell wall stiffening. Cellulase apparently is unable to mechanically alter coleoptiles that have been previously boiled in acetic anhydride in the same manner as those not so treated. This effect of acetic anhydride can be reversed by treatment with 0.1 *N* sodium hydroxide at 23° C for 5 minutes. Treatment with boiling acetic anhydride following cellulase digestion will also reverse the effect of cellulase on extensibility. The difference in extensibility due to IAA treatment was retained through the acetylation and cellulase treatment.

Table 3. *Proline and hydroxyproline distribution in Avena coleoptiles*

Protein fraction	Per cent of tissue dry weight	
	Proline	Hydroxyproline
Extractable protein	0.76	0.010
Cell wall (or residual) protein .	0.08	0.020

Table 4. *Extensibility of sections after sequential treatments with acetic anhydride, sodium hydroxide, and/or cellulase*

Treatment	Extensibility cm × 10³/g	
	Control	IAA
None	1.0	2.3
Acetic anhydride	0.5	1.0
Acetic anhydride Cellulase . .	0.5	1.0
Acetic anhydride 0.1 *N* NaOH	1.0	2.0
Acetic anhydride 0.1 *N* NaOH Cellulase	2.5	5.1
Cellulase	2.5	5.1
Cellulase Acetic anhydride . .	1.3	2.5

Discussion

The mechanical properties of methanol-killed *Avena* coleoptiles have been studied with the aid of a constant rate of extension instrument. These experiments show that it is possible to measure the extensibility of killed *Avena* coleoptile sections by force-extension methods and that treatment of the living tissues with IAA changes this extensibility.

Measurements of cell wall properties obtained using living tissues are often complicated by internal turgor stresses, a fact recognized by Ursprung and Blum (1924) and by Heyn (1933, 1940). Differences in tissue mechanical properties reflecting turgor changes, for example, have imposed a severe limitation upon measurements of wall deformability by bending techniques. Lockhart (1959) has shown that the turgor pressure must remain constant if bending angles are to be related to differences in extensibility. Boiling the tissue in methanol largely eliminates internal stress factors. Our results show that the difference in

extensibility between IAA-treated and non-IAA-treated tissues does not depend on the continued presence of the intact protoplast or internal stress factors.

Evidence that the extensions measured by force-extension methods are independent of a redistribution of material has been demonstrated by OLSON and CLELAND (1964) who showed that the effect of IAA on extension can be detected in IAA-treated but nongrowing sections when the treatment was conducted in isotonic mannitol solution. It has already been established that when sections are restrained from growing in this way, there is still an IAA-induced alteration in the tissue (CLELAND and BONNER, 1956).

Boiling in methanol followed by rehydration and treatment with the protease complex from *Streptomyces griseus* (Pronase) removes over 97% of the nitrogen from coleoptiles. However, over 60% of the hydroxyproline of the whole coleoptile remains in the coleoptile after this treatment. This hydroxyproline is undoubtedly in the hydroxyproline-rich protein that LAMPORT and NORTHCOTE (1960) suggested is an integral part of primary wall of actively growing cells. The data, therefore, show that removal of most of the protein in the coleoptile, including some hydroxyproline-containing protein, does not affect the extensibility. This provides evidence that tissue extensibility is not dependent upon continued presence of the protoplast but is characteristic of the cell wall. The hydroxyproline-rich fraction not removed by this treatment may be structurally important.

Hot dilute mineral acid increases extensibility. According to RAY (1963) and RAY and ROTTENBERG (1964) this treatment removes hemicellulose which might be expected to contribute to the overall mechanical properties of coleoptiles. It is interesting to note, however, that the IAA effect on extensibility is still observable after the hot acid treatment.

The studies involving cellulase and acetic anhydride treatment of coleoptiles show that the extensibility of plant cell walls can be altered by chemical treatment and that force-extension measurements can be used to detect these changes. Acetylation was selected as one chemical modification because of its wide use in studies of cellulose (CONRAD, HARBRINK and MURPHY, 1963), collagen and gelatin (BELLO and BELLO, 1963) structure. Heating coleoptiles in acetic anhydride or mixtures of acetic acid and acetic anhydride acetylates amino groups and, more slowly, hydroxyl groups. In addition, it may introduce crosslinks between carboxyls through anhydride formation. Under the conditions used, the degree of acetylation was sufficient to produce a stiffening of the coleoptiles. By analogy with the corresponding treatment of cotton with acetic anhydride, this stiffening may be the result of increased hydrogen bonding through the formation of new esters. The fact that acetylation essentially stopped the action of cellulase on the coleoptiles is good evidence that

the cellulase substrates, cellulose and hemicellulose, have been altered. The effect of acetic anhydride is reversible both with respect to its inhibition of cellulase action and its effect on force-extension properties. Brief treatment of acetylated coleoptiles with 0.1 *M* sodium hydroxide makes them again susceptible to attack by cellulase, increases their extensibility and decreases their break-load. This treatment would be expected to disrupt the new ester linkages but not amide linkages or anhydride crosslinks.

Bonner (1961) has recently discussed the effect of auxin on the reorientation of cellulose microfibrils of coleoptiles detected under longitudinal stretching. Based on some earlier studies in which plasmolyzed coleoptile tissue was stretched longitudinally under the polarizing microscope (Bonner, 1935), these results show that IAA pretreatment of the tissue markedly reduced the amount of reorientation of cellulose microfibrils in response to extension. In IAA-treated tissue, the interaction between fibrils has been decreased and the ability of the microfibrils to slide past one another has been increased. Thus, it is not surprising that treatments that disrupt cellulose microfibril interaction such as cellulase digestion would also reduce the mechanical strength of the cell wall. The magnitude of the changes introduced by acetylation and deacetylation of cellulose is similar to those introduced by IAA. It is significant that the extensions of dry coleoptiles also show the effects of prior IAA treatment. In dry coleoptiles there may be more secondary bonds than in wet ones, as evidenced by the appearance of yield points in the extension curves and higher break-loads. The additional secondary bonds between the polymers of dry coleoptiles may follow as a result of the collapse of the tissue on removal of water (Morton and Hearle, 1962). That the IAA effect remains detectable under these conditions suggest the polymers themselves have been altered. The evidence presented in this paper agrees with the findings of Bonner (1935). In addition, it suggests that the changes responsible for the decrease in interaction between fibrils involve cellulose and hemicellulose. The extent of the chemical changes that result in altered mechanical properties may be very small.

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