In Vitro Autolysis of Plant Cell Walls'
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Summary. Primary cell walls of Zea mays prepared in a glycerol medium are capable of autolysis in vitro. Autolysis results in solubilization of about 10% of the wall substance during an 8 hour incubation period. Approximately 10% of the solubilized material is glucose and the remainder consists of an unidentified polymer which yields only glucose upon hydrolysis. Cell wall autolysis is a linear function of time of incubation and of wall concentration. The autolytic process occurs optimally over the pH range of 5.5 to 6.5. The possible relationship between autolytic capacity and capacity for elongation is discussed.

Autolytic degradation of cell walls during the course of normal growth and development of bacteria (1,17), fungi (11), and higher plants (13) is well established. For example, in higher plants, formation of laticiferous vessels requires autolysis of the end cell walls so as to form a continuous tube. More important, however, for purposes of the present work, is the fact that degradative processes do occur in cell walls during extension growth of young cells (9,10), and thus are probably part of the normal growth process.

As a working hypothesis, we have found it convenient to visualize coleoptile growth as occurring in 2 stages. In the early stage of cell division, primary wall synthesis predominates. In the stage of elongation, wall autolysis and breakage of cross-linkages in the hemi-cellulosic matrix might be expected to occur. In tissues other than a coleoptile a final stage of maturation would result in secondary thickening. If the above view is correct, then it becomes important to study degradative reactions of the cell wall in greater detail. We wish to report in the present work that the primary wall of young corn coleoptiles will autolyze in vitro resulting primarily in the solubilization of a non-reducing polysaccharide. To the best of our knowledge this, and a previous short communication (5), are the first reports of in vitro plant cell wall autolysis.

Materials and Methods

Coleoptiles were excised from seedlings of either Michigan Hybrid 300 field corn or Stowell's Ever-

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green Hybrid sweet corn after 5 days of germination at 25° and 50% relative humidity. After excision, the coleoptiles (without leaves) were chilled to 1° and then stored at −20° until a sufficient weight, usually 100 g, had been collected. Glycerol was used as the homogenization medium and purification of the walls accomplished by filtration of the glycerol suspension through a glass bead filter at 1°, as previously described (3). Collection of the final pellet, containing the cell wall fragments and glycerol, was accomplished by centrifugation for 60 minutes at 73,000 × g. This glycerol pellet may be stored for about 2 weeks at −20°. Storage for periods as long as 1 month results, however, in a loss of autolytic activity. For convenience in handling, glycerol was removed from the pellet by extraction with solvents at low temperatures. Approximately 5 g of glycerol cell wall pellet containing 0.3 g of dry cell wall were suspended in 10 ml of absolute ethanol at −10 to −20°. The cell walls were collected by centrifugation for 3 minutes at 1000 × g. This process was repeated 3 times maintaining the temperature at −10 to −20°. The pellet was then suspended in dry acetone at −20° and again collected by centrifugation. The walls were resuspended in dry diethyl ether, collected by centrifugation and dried in vacuo in a dessicator. Final drying was accomplished, in vacuo, over P₂O₅ for 12 hours at room temperature. The resultant white granular powder is free of intact cells and was stored at −20° over anhydrous calcium sulfate. The usual yield is 1 g and this dry preparation is stable for at least 3 months. It is important that the initial homogenization and filtration be accomplished quickly. It is also important that all subsequent steps be conducted at low temperatures and under dry conditions since traces of solvent or of water cause loss of the autolytic capacity.
All reagents used were of the highest purity commercially obtainable. Glycerol was freshly distilled in vacuo prior to use. A cellulose preparation (Alphacel) was obtained from Nutritional Biochemicals. Reducing sugars were measured by the method of Nelson (12), and glucose by glucose oxidase (Worthington Biochemical Corporation). Chromatography of hydrolysis products was performed by the descending technique using Whatman No. 1 paper and pyridine-ethyl acetate-water (2:8:1) (16) or butanol-acetic acid-water (4:1:5) (15) as solvents. Sugars were detected with silver nitrate reagent or with aniline phthalate (15). Partial hydrolysis of the polymeric carbohydrate, solubilized during autolysis, was accomplished with 4% nitric acid at 100°C (2).

Results

Cell wall autolysis was obtained by suspending a weighed quantity of dried cell wall in buffer or distilled water and incubating for the indicated period of time at 37°C. The reaction was terminated by boiling for 5 minutes, and the residual wall preparation collected by filtration through tared Whatman No. 540 ashless filter paper discs, or through Millipore filters dried to constant weight. The loss of weight of the wall due to incubation was determined by means of a Cahn micro-electrobalance. Reducing sugars and solubilized polymeric material were then assayed in the filtrate. A zero time control was used for each reaction condition. This was obtained by heating the wall preparation to 100°C for 5 minutes immediately after adding the suspending medium. The boiled control was then incubated under the same conditions as the experimental sample.

A typical experiment is illustrated by the data of Table I. As can be seen 22% of the wall dry weight becomes solubilized during incubation followed by boiling. A 9% weight loss occurs in the boiled control, and thus approximately a 13% weight loss may be attributed to enzymic autolysis. The 9% weight loss in the boiled control consists, mainly of residual glycerol in the wall preparation and, in part, of loss of cell wall material which is soluble in hot water but not in glycerol. The control weight loss may be reduced by termination of the reaction with 5% cold trichloroacetic acid. Use of trichloroacetic acid, however, interfered with subsequent chromatographic analysis.

The data of Table II illustrate the composition of the material solubilized during autolysis, and which thus appear in the filtrate. Enzymatic activity results in a 10% loss of the initial dry weight of the cell wall. Of this solubilized material 9% consists of a reducing sugar. Chromatography of aliquots of the filtrate showed almost all of the reducing sugar to be glucose with trace amounts of arabinose and xylose. No mannose or fructose was found. Of the remaining substances, other than contaminating glycerol, 91% of the enzymatically solubilized components consisted of an, as yet, unidentified non-reducing polymer. The bulk of the polymer was of sufficiently low molecular weight to pass through dialysis tubing in 16 hours. Both the dialyzable and non-dialyzable fractions were hydrolyzed only 10% during 4 hours at 100°C in 4% nitric acid. Only glucose could be detected in the acid hydrolysate by chromatography and quantitative analysis for glucose in the hydrolysate, using glucose oxidase, accounted for 96% of the reducing sugar as determined by the Somogyi-Nelson method.

Table I. Weight Loss of Cell Walls and of Alphacel due to Incubation and to Boiling

<table>
<thead>
<tr>
<th>Treatment of cell wall or alphacel</th>
<th>Loss in initial dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Boiled (5 min); without incubation</td>
<td>5%</td>
</tr>
<tr>
<td>2) Boiled before incubation</td>
<td>9%</td>
</tr>
<tr>
<td>3) Boiled after incubation</td>
<td>22%</td>
</tr>
<tr>
<td>4) Incubated and reaction terminated by filtration</td>
<td>17%</td>
</tr>
<tr>
<td>5) Alphacel boiled after incubation</td>
<td>5%</td>
</tr>
</tbody>
</table>

Fig. 1. Time course of cell wall autolysis. Each tube contained 50 mg of cell wall material in 2.5 ml of water. Incubation was for the indicated time at 37°C.
Table II. Autolytic Weight Loss of Cell Walls

Incubation was for 8 hours at 37° using 100 mg of cell wall in a total volume of 5 ml. Weight loss of the walls was determined gravimetrically. Glycerol was identified by chromatography and estimated by difference. Reducing sugar was determined as described in the text. Non-dialyzable polymer was estimated gravimetrically after lyophilization of the contents remaining in the dialysis bag. Dialyzable polymer was estimated by a lyophilization of the water exterior to the dialysis bag, then extracting glycerol with water, redrying, and weighing the retrograded polymer.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Boiled after incubation</th>
<th>Boiled before incubation</th>
<th>Difference</th>
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</thead>
<tbody>
<tr>
<td>Residue recovered after incubation</td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>In filtrate:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>80</td>
<td>91</td>
<td>—11</td>
</tr>
<tr>
<td>Reducing sugar</td>
<td>7</td>
<td>7</td>
<td>0.0</td>
</tr>
<tr>
<td>Non-dialyzable polymer</td>
<td>1.2</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Dialyzable polymer</td>
<td>2.6</td>
<td>0.5</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Although most of the weight loss of the cell wall was due to solubilization of a polysaccharide polymer, difficulties in hydrolyzing the polymer and thus, lack of a convenient assay, led us to use the appearance of reducing sugar in the filtrate as a measure of wall autolysis. In experiments not here reported, it was found that the appearance of glucose in the filtrate correlated well with loss in weight of the wall. As shown in figure 1, the time course of autolysis is linear during, at least, an 8 hour period. The progress of the reaction with time was the same when toluene was added to the reaction mixture to preclude any possible microbial activity. The linearity of the reaction and the insensitivity to toluene demonstrates that microbial activity does not contribute to the autolytic reaction.

The amount of reducing sugar appearing in the filtrate is a linear function of the amount of wall material used (fig 2). Since the cell wall preparation served both as the enzyme system and as substrate, it is impossible, with intact walls, to separately determine the effect of enzyme and of substrate concentration. Nonetheless, the data indicate that over, at least, the indicated ratios of wall weight to suspending medium, comparison of experiments using differing amounts of wall tissue is possible.

Figure 3 shows the effect of pH on the rate of wall autolysis. Since the reaction is linear for at least 8 hours, these data do, in fact, represent rates. The optimum pH is apparently between 5.5 and 6.5.

**Discussion**

The present data indicate that primary cell walls of corn coleoptiles do contain enzymes capable of autolyzing the walls. This is the first report of in vitro autolysis of plant cell walls. It is of course difficult to prove that the observed autolysis is not due to the presence of soluble cytoplasmic enzymes which are adsorbed to the wall during homogenization and which are not eluted by the preparative methods here employed. However, the possibility of cytoplasmic contamination of the walls is unlikely. For example, the absence of particulate contamination of cell walls prepared by the procedure employed has been previously shown, using both the light microscope (3), and electron microscope (6). Similarly, the efficacy of the glycerol washing procedure has been studied by deliberate contamination of the walls with a soluble enzyme and subsequent repurification (4). Such experiments result in quantitative removal of the contaminating enzyme. There is, in addition a large body of data demonstrating the presence of...
protein and of various synthetic and hydrolytic enzymes in the primary cell wall (7), and significantly, the unique presence of hydroxyproline-rich proteins in the wall (8). In addition compelling evidence for the presence of enzymes involved in wall polysaccharide material has been provided by Setterfield and Bayley (14). These workers demonstrated, by means of radioautography, that ¹⁴C labeled glucose was uniformly incorporated throughout the primary wall. This result would not be possible if polysaccharides were synthesized within the protoplast and then secreted into the wall. Thus, we feel, that the presence of both hydrolytic and synthetic enzymes, as components of the primary cell wall has been established.

The question of what components of the wall are hydrolyzed or changed in physical properties during extension growth is not settled. Lamport (7) has focused attention on the hydroxyproline-rich cell wall proteins. Changes in this fraction to alter structural rigidity would form a nice analogy to the structural proteins of animal tissue. MacLachlan and Young (9) and MacLachlan and Duda (10) have demonstrated with in vivo experiments that cell walls are autolyzed, and have studied the activity of cellulase. Our results point, however, to hydrolysis or change in degree of branching of a hemi-cellulosic polysaccharide. This polysaccharide has, so far, yielded only glucose upon hydrolysis and, on the basis of resistance to acid hydrolysis, could be a 1-3 polyglucan. Because of the resistance of the solubilized polymer to acid hydrolysis, and because in experiments not here reported, we have observed autolysis of up to 34% of the wall, when cellulose constitutes only 25% (3) of the wall and protein only 5% (3), we adopt as a working hypothesis that changes in a polyglucan are involved in extension growth. Further work is required to determine which cell wall components are altered during extension growth and to determine if a correlation exists between the capacity for extension growth and the capacity for in vitro autolysis.

Addendum

A communication by Y. Masuda and S. Wada (Bot. Mag., Tokyo 80, pages 100-02, 1967) reports that beta-1,3-glucanase treated oat coleoptile sections elongate at a rate commensurate with IAA treated sections.

Literature Cited