The Effects of pH, Ionic Strength, and Ethylene on the Extraction of Cellulase from Abscission Zones of Citrus Leaf Explants

Received for publication September 16, 1974 and in revised form January 13, 1975

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ABSTRACT

The solubility of cellulase extracted from the abscission zones of citrus leaf explants (Citrus sinensis L. Osbeck) in sodium phosphate buffer depends on the pH of the extracting solution and, to a lesser extent, on the ionic strength. By increasing molarity from 0.01 to 0.16, the solubility of cellulase increased from 51% to 89% at pH 6.1 and from 70% to 98% at pH 7. In all cases, residual cellulase was further extracted from the pellet by buffer containing 1 M NaCl. Most of the enzymic activity was found in tissues proximal to the separation line, and activity of the cellulase which was soluble in phosphate buffer was closely correlated with abscission at both pH values. When extraction of cellulase at pH 6.1 with phosphate buffer was followed by a reextraction of the pellet with buffer containing 1 M NaCl, the activity of the cellulase soluble in the fortified buffer was also correlated with abscission. Pretreatment of explants with ethylene increased the solubility of cellulase in the phosphate buffer regardless of the pH used at the first extraction.

Moreover, can reextraction of the pellet with buffer containing 1 M NaCl promote further extraction of a specific cellulase fraction? Does ethylene affect the extractability of cellulase by the phosphate buffer?

The present investigation attempts to provide answers to these questions.

MATERIALS AND METHODS

Plant Material. Four- to 10-month-old leaves were picked from 40-year-old Shamouti orange (Citrus sinensis L. Osbeck) trees and transferred to the laboratory in moist chambers. Twenty-mm long explants (10 mm from the petiole and 10 mm from the midrib of the leaf blade) were excised and washed in running tap water. Petioles of 10 explants were tied to a glass slide and four such groups per treatment were kept for the desired period in 12-liter containers in the dark at 25 C. The containers were connected to a humidified flow system with either 10 μl/l of ethylene or air. The concentration of ethylene in the containers was determined routinely through the analysis of an air sample by means of gas chromatography (Packard Model 7400). Each experiment was repeated at least three times and the percentage of abscission was determined by counting the distal sections that had already abscised and those that abscised as a result of a gentle touch administered by forceps (13). Explants were then taken for cellulase assays.

Extraction Procedures. One-mm sections from the distal and proximal sides of the separation line were sampled separately from 40 explants, and the sections from each side were pooled separately for cellulase analysis.

Cellulase soluble in buffer. This cellulase was usually extracted by means of Ultra turrax (24,000 rpm) in 15 ml of 0.05% cysteine in sodium phosphate buffer at either pH 6.1 and 20 mM (procedure A) or at pH 7 and 0.1 M (procedure B). In both procedures the homogenate was extracted for 15 min on a magnetic stirrer at 2 to 4 C and centrifuged at 15,000g (procedure A) or 5,000g (procedure B) for 10 min at 4 C (IEC centrifuge, Model B-35). The supernatant fluid was decanted and the pellet was reextracted twice and centrifuged again as described above. When not otherwise stated, activity was determined in the combined first two extracts. The third extract was discarded since the activity detected in this washing extract was negligible.

Cellulase soluble in buffer containing NaCl. Pellets resulting from procedures A and B were further extracted in 20 mM phosphate buffer (pH 6.1) containing 1 M NaCl, according to the method of Lewis and Varner (8). The homogenate was then centrifuged as described above at 5,000g, the pellet was dis-
carded, and the supernatant fluid was assayed for its cellulase activity.

**Gel Electrophoresis.** Electrophoresis of cellulase obtained by both extraction procedures was done as described by Davis (4) and Ornstein (11). Protein was precipitated after the addition of anhydrous (NH₄)₂SO₄ to yield 80% concentration (w/v), and was separated after 1 hr by centrifugation at 15,000g for 10 min. The protein was resuspended in 0.1 M sodium phosphate buffer at pH 7, and then was dialyzed overnight against the same buffer diluted 10 times and centrifuged as above. Aliquots of 0.1 ml (200–300 µg of protein) were subjected to electrophoresis on 8% polyacrylamide gels (0.6 × 10.2 cm) at 5 mamp/tube in the dark for 110 min. After the front had migrated approximately 8.5, the gels were frozen and subsequently cut into 2.5-mm slices. Cellulase was extracted from the slices by shaking them overnight in 1 ml of 20 mM sodium phosphate buffer at pH 6. All manipulations were done at 4°C. The buffer extract was then assayed for its cellulase activity.

**Cellulase Assay.** Cellulase activity was tested as previously described (13). Changes in the viscosity of the enzyme-substrate mixture (0.5 ml of extract in 10 ml of 1.1% carboxymethylcellulose, sodium salt (BDH) in 22 mM phosphate buffer, pH 6) was determined by means of an Eeca viscosimeter (size 300). Extracts boiled for 10 min were used as controls.

**Cellulase units.** For calculation purposes, 100 units were arbitrarily defined as the amount of enzyme causing a decrease of 21% in the flow time of the enzyme-substrate mixture after 18 hr of incubation at 37°C in the dark (13). Attention was paid that the recorded rate of decrease in flow time during the assay period should be in the linear portion of the hyperbolic function. It was also established that enzymic activity remained equally stable for the whole experimental period.

Protein analyses were carried out on aliquots of extracts following the methods of Lowry et al. (10).

**RESULTS**

The following experiments were designed to find out if more cellulase can be extracted by a buffer containing 1 M NaCl following extractions in 0.1 M phosphate buffer at pH 7. Leaf explants were exposed for 45 hr to 10 µl of ethylene. Cellulase extraction from the abscission zone was performed at 90% abscission. Results in Table 1 show that 88% of total cellulase and 97% of the protein were extracted in the course of the first two extractions in phosphate buffer. Additional cellulase was extracted from the pellet by the fortified buffer. Although much less protein was extracted by the buffer containing 1 M NaCl, its specific cellulase activity was much higher than that found in the soluble fraction of cellulase extracted without the addition of NaCl.

The effect of the ionic strength of phosphate buffer at pH 6.1 and 7 (comparisons of procedures A and B) on cellulase solubility was studied further in a similar experimental system (Figs. 1 and 2). It is evident that at pH 6.1 (procedure A, Fig. 1) the molarity of the buffer affected the amount of extractable soluble cellulase obtained from both proximal and distal sections. The increase in activity of cellulase soluble in buffer, caused by the increase in the molarity of the buffer, was accompanied by a marked decrease in the level of cellulase activity in the buffer containing 1 M NaCl. It was also found that when sections from the distal tissue were extracted at pH 6.1 (procedure A, Fig. 1), only a small portion of cellulase soluble in buffer was extractable, even when the molarity of the buffer was increased to 0.16 M. The extraction of cellulase soluble in buffer was much more efficient at pH 7, and no effect of the molarity was observed at the range of 20 mM to 0.16 M (procedure B, Fig. 2). Again, the activity of cellulase soluble in buffer, which was extracted from tissue proximal to the separation line, was higher than in the tissue distal to it.

The extraction of the residual cellulase, by phosphate buffer containing 1 M NaCl, from the proximal and distal tissue sections was closely related to the pH; activity was much lower when the extraction of plant material was done at pH 7 than when done at pH 6.1 (Figs. 1 and 2). However, in both cases the activity of cellulase soluble in buffer containing 1 M NaCl, on both sides of the separation line, was similar. Nevertheless, the molarity of the buffer exerted a slight effect on the amount of cellulase extracted with the fortified buffer, namely, when ionic strength increased to 0.16 M less activity could be detected.

The correlation between abscission and the activity of both cellulases was studied in explants which were exposed to air up to 96 hr from excision, and at 100% abscission. The determination of enzymic activity started at 10% abscission, 48 hr from excision. Extraction of cellulase soluble in buffer was done by the two procedures; residual cellulase soluble in buffer con-

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**Table 1. Distribution of Cellulase and Protein Soluble in Phosphate Buffer, and Cellulase Soluble in Buffer containing 1 M NaCl (Procedure B)**

Two hundred explants were exposed to 10 µl of ethylene for 45 hr and reached 90% abscission.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total</th>
<th>Phosphate buffer</th>
<th>1st extraction</th>
<th>2nd extraction</th>
<th>4th extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 7, 0.1 M, −NaCl</td>
<td>pH 6.1, 20 mM</td>
<td>pH 6.1, 1 M NaCl</td>
<td></td>
</tr>
<tr>
<td>Cellulase (units)</td>
<td>44,375</td>
<td>24,375</td>
<td>14,375</td>
<td>5,625</td>
<td></td>
</tr>
<tr>
<td>Protein (mg)</td>
<td>84</td>
<td>67.5</td>
<td>14</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Specific activity (units/mg protein)</td>
<td>528</td>
<td>370</td>
<td>1,000</td>
<td>2,125</td>
<td></td>
</tr>
</tbody>
</table>

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**Fig. 1. Effect of ionic strength on the solubility of cellulase in phosphate buffer at pH 7, extracted from the proximal and distal sides of the separation line.** Explants were exposed to 10 µl.1 of ethylene for 35 hr and reached 75% abscission. Total cellulase activity (△); activity of cellulase soluble in buffer (●); activity of cellulase soluble in buffer containing 1 M NaCl (○).
taining 1 M NaCl was extracted thereafter in the routine way.

Results in Figures 3 and 4 show that the activity of cellulase soluble in the fortified buffer was correlated with abscission only when using procedure A. Moreover, the activity of cellulase soluble in the fortified buffer was higher when this procedure was employed. However, the level of activity of cellulase soluble in buffer containing 1 M NaCl was similar on both sides of the separation line, regardless of the procedure used (Figs. 3 and 4). As expected from previous works (5, 13), substantial cellulase activity was found at the abscission zone even at 10% abscission. In Citrus, contrary to bean explants (8), a positive correlation was found between the activity of cellulase soluble in buffer and abscission, even when procedure A was used.

In the following, we investigated the possibility that ethylene may affect the solubility of the cellulase complex in the phosphate buffer, and that this effect may be responsible for the correlation just mentioned. Table II clearly shows that more activity of cellulase soluble in buffer was found when explants were previously exposed to ethylene. These results were obtained with both procedures and in sections from both sides of the abscission zone, but the ethylene effect was much stronger at pH 6.1.

The data related to total cellulase activity, presented above, clearly showed quantitative differences between proximal and distal sections (Figs. 3 and 4). Figure 5 presents zymograms of cellulase isozymes which were obtained by polyacrylamide gel electrophoresis. These zymograms show a marked increase in cellulase activity during abscission, as was already found by measuring total activity (Figs. 3 and 4), and reveal no significant qualitative changes in the pattern of cellulase isozymes on either sides of the separation line. Similar zymogram patterns were obtained when the extracting buffer at pH 7 and 0.1 M also contained 1 M NaCl.

**DISCUSSION**

The present study clearly shows that when cellulase was extracted from the abscission zones of ethylene-treated citrus leaf explants at pH 7 (procedure B) more than 90% of the activity were found in the phosphate buffer. The effect of the ionic strength was secondary, since the increase in the molarity was less effective than the increase in pH. The percentage of cellulase activity in the buffer at 10 mM to 0.16 M and pH 6.1 was similar to that found at pH 7 and 20 mM. Nevertheless, a further increase in the molarity at the higher pH level increased to some extent the activity of cellulase soluble in buffer. It is evident that at least in citrus, our previous extraction procedure (5, 13), herein designated procedure B, should be recommended.
Table II. Effect of Ethylene on Extraction of Cellulase from Proximal and Distal Sections

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proximal sections</th>
<th>Distal sections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
<td>Ethylene</td>
</tr>
<tr>
<td></td>
<td>1st + 2nd extractions</td>
<td>4th extraction</td>
</tr>
<tr>
<td>pH 6.1, 20 mm, -NaCl</td>
<td>5370</td>
<td>34.6</td>
</tr>
<tr>
<td>pH 6.4, 20 mm, -1 M NaCl</td>
<td>7302</td>
<td>85.1</td>
</tr>
</tbody>
</table>

Proximal sections
Air
Ethylene
Distal sections
Air
Ethylene

Fig. 5. Polyacrylamide gel electrophoresis of cellulase extracted by phosphate buffer at pH 7 and 0.1 m from the distal and proximal sections of leaf explants in air. Arrows indicate running front.

because at the lower pH and the weaker ionic strength (8) (procedure A) only 60% of total cellulase is extracted by the buffer (Figs. 1 and 2).

Reid et al. (14), who used ethylene-treated explants of Phaseolus vulgaris, were able to extract only 21% of the cellulase soluble in phosphate buffer, at pH 6.1 and ionic strength of 20 mm. In citrus leaf explants some cellulase remained bound to the plant material after the first extraction in buffer at pH 7, and could be extracted only when the buffer was enriched with 1 m NaCl (Table I and Fig. 2). The fact that the specific activity of the enzyme at this fraction was high (Table I) indicated that a much greater portion of the protein in the buffer containing 1 m NaCl extract was cellulase protein.

The present investigation confirmed earlier data (5, 13) that the activity of cellulase soluble in buffer in citrus is closely correlated with abscission, and that this activity is more evident in the tissue proximal to the separation line. It also demonstrated that contrary to bean explants, the same is true when ethylene is both present or absent and when procedure A was used (Figs. 3 and 4). However, when cellulase was extracted at a lower pH and molarity level, abscission and the activity of cellulase soluble in buffer containing 1 m NaCl were correlated. This may be attributable to the fact that under such conditions a significant portion of the enzyme was still bound to the plant material and could only be extracted with the fortified buffer. Lewis and Varner (8) and Reid et al. (14) suggested that the cellulase 4.5 isozyme in bean explants' abscission zone was extracted primarily by the buffer, and that the major portion of the cellulase 9.5 was extracted only after the addition of NaCl. Such results could lead to a conclusion that the nature of the soluble and residual cellulase is determined by differences in their isoelectric points and other properties. However, results in Figure 5 revealed that three cellulase isozymes in the abscission zones of citrus leaves on polyacrylamide gels when the tissue was extracted at pH 7 with 0.1 m buffer only, and they demonstrated a similar nature when the cellulase complex was extracted in the presence or absence of NaCl. In a recent study with Phaseolus vulgaris, Linkins et al. (9) changed procedure A and showed that both the acidic and the basic cellulase isozymes could be extracted at pH 6.1 if molarity is brought up to 0.2 instead of 0.02 and is in the presence of 1 m NaCl. This was also true when molar NaCl was 20 mm and 1 m NaCl was added to the extractive medium (14). These findings suggest that the isozymes be better defined by their specific chemical-physical properties (9, 14) rather than by the former terms, soluble and residual cellulases (8).

The fact that ethylene plays a dual role in abscission is well documented (2, 3, 12). It promotes, and even is required for, the process of aging; it induces the enzymes required for cell separation. It is also suggested that ethylene controls the secretion or release of cellulase to the cell wall (3). This was also found to be true in the abscission zone of mature Valencia orange fruits (12). It is now suggested (9, 14) that only cellulase 9.5 isozyme is closely related to ethylene and involved in the abscission process, whereas the increased activity of cellulase 4.5 is auxin induced and is related to processes of cell-wall loosening required for growth. The recent report of Reid et al. (14), that ethylene induced the increase of cellulase 9.5 in Phaseolus vulgaris explants, and our findings that ethylene may also influence the amount of cellulase which can be extracted by the buffer (Table II), suggest the hypothesis that ethylene may also affect the chemical-physical properties of cellulase isozymes which are hitherto characterized by their solubility, or changes the enzyme binding properties of the particulates in the homogenate.

LITERATURE CITED

changes in the stem and petiole anatomy and cellulase enzyme patterns in *Phaseolus vulgaris* L. Plant Physiol. 52: 554-560.


