Cell Wall Free Space of Cucumis Hypocotyls Contains NAD and a Blue Light-Regulated Peroxidase Activity

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ABSTRACT

Solutions were obtained from the cell wall free space of red light-grown cucumber (Cucumis sativus L.) hypocotyl sections by a low-speed centrifugation technique. The centrifugate contained NAD and peroxidase but no detectable cytoplasmic contamination, as indicated by the absence of the activity of glucose-6-phosphate dehydrogenase from the cell wall solution. Peroxidase activity centrifuged from the cell wall of red light-grown cucumber hypocotyl section could be resolved into at least three cathodic isoforms and two anodic isoforms by isoelectric focusing. Treatment of red light-grown cucumber seedlings with a 10-minute pulse of high-intensity blue light increased the level of cell wall peroxidase by about 60% and caused a qualitative change in the anodic isoforms of this enzyme. The increase in peroxidase activity was detectable within 25 minutes after the start of the blue light pulse, was maximal at 35 minutes, and declined to control levels by 45 minutes of irradiation. The inhibitory effect of blue light on hypocotyl elongation was more rapid than the effect of blue light on total wall peroxidase activity, leading to the conclusion that growth and peroxidase activity are not causally related.

Peroxidase, extracted from cell walls by various methods, has been shown to vary in activity with hormone-stimulated growth and naturally occurring gradients of growth rate (3, 12, 13, 18, 19, 23). An inverse relationship between growth rate and cell wall peroxidase activity has been found. One model for peroxidase action holds that cell wall extensibility can be regulated by the formation of intermolecular cross-links between wall polysaccharides or proteins via phenolic components such as ferulic acid or tyrosine residues (8, 17). Cross-linking structural wall polymers could alter cell wall extensibility (6, 8, 10), and intermolecular tyrosine cross-links (7) could affect the function of cell wall proteins as well as their structure.

Because growth rates change slowly, while metabolic processes are also changing, a unique connection between peroxidase activity and growth has not been demonstrated. Even extracting peroxidase from the CWFS to obtain rapidly changing pools of enzymes has yielded ambiguous results. CWFS peroxidases have been obtained from several tissues exhibiting changes in growth rate, by treatment of intact stem segments with vacuum infiltration followed by low-speed centrifugation (3, 14, 15, 18, 23), but no consistent pattern has been found. In pea stems showing an auxin-induced increase in elongation, a decrease in CWFS peroxidase activity was observed (18). However, in corn coleoptiles in which red light increases elongation rate, the peroxidase activity extracted from the CWFS also increased instead of decreasing as expected (14). Hypocotyls of light-grown Sinapis show decreasing activities in specific isozymes in tissues showing increased growth rates, but the enzyme activity changes were slower than the changes in growth rate (3).

We have utilized the rapid inhibition of stem elongation in Cucumis seedlings as a system to further test the hypothesis that changes in cell wall peroxidase activity mediate changes in stem elongation rate. In Cucumis hypocotyls, the growth rate is inhibited by 50% within 5 min of irradiation with high-intensity BL, and recovery from growth inhibition is also rapid (5, 21). Although the mechanism does not appear to be straightforward, Cosgrove (6) determined that BL interferes with cell wall loosening, not alterations in turgor. Peroxidase involvement in the BL-induced inhibition of stem elongation is suggested by evidence that ascrobate prevents the BL response in cucumber and the demonstration that ascorbate inhibits the activity of a CWFS peroxidase from the same tissue (21). Centrifugates from cucumber hypocotyl CWFS (18, 23, 24) were assayed for peroxidase and the presence of the cofactor NADH, which is required for the peroxidase-mediated reactions that are postulated to contribute to the regulation of cell wall expansion (17). Our data show that, whereas peroxidase and NAD are present in the cell wall of cucumber hypocotyls and could participate in inter- or intramolecular bond formation, the changes in growth rate induced by BL do not correlate in time with changes in peroxidase activity.

MATERIALS AND METHODS

Plant Material

Growth conditions, light sources, and light measurements were as described previously (21). Cucumber seedlings (Cuc-

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Abbreviations: CWFS, cell wall free space; BL, blue light; G6PDH, glucose-6-phosphate dehydrogenase; IEF, isoelectric focusing.
umis sativus L. cv Burpee's pickler) were grown in clear plastic boxes under dim red light for 4 d. Plants were handled under dim red light during harvesting and preparation for centrifugation of cell wall solutions. For experimental BL treatments, a box of 300 to 400 seedlings was placed between two banks of blue fluorescent lights and irradiated for 10 min. The average fluence rate delivered to the plants was 100 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \). Harvesting (cutting and loading of sections) began immediately after the end of the BL pulse.

**Centrifugation of Hypocotyl Sections**

Previously developed techniques (18, 24), with minor modifications, were used to centrifuge solutes from the free space of hypocotyl sections. Hypocotyls were harvested using a BioCorp homogenizer and used to prepare the cell wall fraction. The central region of the gel created by overlaying the gel with a plastic strip with holes sufficient to hold 15 \( \mu \text{L} \) of sample. The small sample size required concentration of the peroxidase from the CWFS. This was accomplished by first desalting the CWFS solution by passing it through a Sephadex PD-10 column and eluting it with 0.1 mM Tris buffer, pH 7. The fractions showing peroxidase activity were pooled and concentrated 100-fold by vacuum evaporation using a Savant Speed-Vac. More than 50% of the original activity was recovered in the concentrated sample. These samples were applied to the IEF gel. Gels were stained for peroxidase using benzidine (1) or 3-amino-9-ethyl carbazole (13).

**IEF**

Prefabricated Servalyt IEF gels, pH range 3 to 10, were used as described previously (2). Samples were applied to wells in the central region of the gel created by overlaying the gel with a plastic strip with holes sufficient to hold 15 \( \mu \text{L} \) of sample. The small sample size required concentration of the peroxidase from the CWFS. This was accomplished by first desalting the CWFS solution by passing it through a Sephadex PD-10 column and eluting it with 0.1 mM Tris buffer, pH 7. The fractions showing peroxidase activity were pooled and concentrated 100-fold by vacuum evaporation using a Savant Speed-Vac. More than 50% of the original activity was recovered in the concentrated sample. These samples were applied to the IEF gel. Gels were stained for peroxidase using benzidine (1) or 3-amino-9-ethyl carbazole (13).

**RESULTS**

**Peroxidase and NAD Content of Cell Wall Centrifugate**

Free space extracts from excised sections of red light-grown cucumber hypocotyls yielded approximately 2 \( \text{mL} \) of solution containing approximately 37 \( \mu \text{g} \) protein/\( \text{mL} \) (Table I). The centrifugate contained peroxidase but no measurable G6PDH activity (Table I). The cell wall solution also contained appreciable levels of NAD (5.9 nmol/mg protein, averaging control and plus BL values) but no measurable NADP. For all enzyme and cofactor assays, total protein in each sample assayed varied by a factor of two or less. This was accomplished by diluting the crude homogenate to achieve protein concentrations in the same range as those found in the CWFS samples. Enzyme and cofactor levels measured were at least 10-fold higher than the lower limit of detectability for each assay.
Comparison of the levels of cytoplasmic components in centrifugates and homogenates of cucumber hypocotyls provides evidence that little or no cytoplasmic leakage into the CWFS fraction occurred. Whereas the centrifugate from hypocotyl did not contain measurable levels of G6PDH or NADP, homogenates contained both G6PDH and NADP as well as peroxidase and NAD (Table I). Experiments were performed to check for phosphatase activity that would convert NADP into NAD by measuring NADP recovery. No such activity was found in either the CWFS or the extract derived from it, and NADP recovery was in the range of 90% (data not shown). When expressed on a per milligram of protein basis, the NAD concentrations in centrifugates and homogenates were similar, despite the fact that the total amounts of protein present in the two preparations differed by several orders of magnitude (Table I). This similarity was probably coincidental.

Comparison of Peroxidase from CWFS with Ionomically Bound Cell Wall Fraction

Total amounts of peroxidase and protein obtained from Cucumis CWFS were consistent with previous findings using other species (18, 23) but considerably less than that reported for protein and peroxidase ionically bound to cell walls (12). Ionically bound cell wall proteins and a cytoplasmic fraction were extracted as described previously (9, 19) and assayed for peroxidase. The results shown in Table I confirm that there is more peroxidase ionically bound to the cell wall than there is in the free space. There is also a significant amount of peroxidase in the cytoplasmic remnant, and the specific activity is similar to that obtained for the crude homogenate (calculated as 24.7 versus 39 nkat/mg protein). Membrane and particulate components of the crude homogenate were removed when producing the cytoplasmic fraction. This may account for the difference between the results for crude homogenate and cytoplasmic remnant fractions.

Effect of BL on Peroxidase Activity and Growth

The procedure for BL treatment of intact seedlings, for cutting and loading hypocotyl sections, and for infiltrating and centrifuging the sections to extract the cell wall solution is shown in Figure 1A. A 10-min exposure of intact cucumber seedlings to 100 μmol m⁻² s⁻¹ BL results in a 50% decrease in growth rate (21) relative to controls maintained in red light. This treatment induced a 60% increase in cell wall peroxidase, expressed as total peroxidase (Table I) or as peroxidase activity per milligram of protein (Table II) relative to red light controls. The amounts of protein and NAD/NADP extracted from cell walls were unaffected by BL. To compensate for small variations between cell wall extracts, NAD levels were expressed on a per protein basis, as were peroxidase results shown in Table II. BL had no effect on peroxidase activity or amounts of NAD/NADP in homogenates of cucumber hypocotyl sections (data not shown).

Analysis of the centrifugates from red light-grown and BL-irradiated hypocotyls by IEF showed that the three cathodic isoforms of peroxidase from the two treatments were similar but that the anodic isoforms were qualitatively different (Fig. 2). Previous experience with this technique indicates that differences in stain intensity are probably not quantitative; therefore, to maximize visibility of qualitative differences, peroxidase was concentrated as much as possible, and equal amounts of peroxidase on an activity basis were loaded into each gel lane. The cell wall solution centrifuged from red-light-grown cucumber hypocotyl sections was centrifuged into carriers for centrifugation was either 20 (A) or 10 min (B). The duration of the cut and load period was controlled and used as an experimental variable. A second experimental variable was a 10- (C) or 30-min (D) delay between the end of irradiation and the beginning of the cutting of sections. In these cases, the cut and load period was 20 min.

**Figure 1.** Schematic for extraction of cell wall solution from Cucumis seedling hypocotyl sections. The time required to cut sections and load them into carriers for centrifugation was either 20 (A) or 10 min (B). The duration of the cut and load period was controlled and used as an experimental variable. A second experimental variable was a 10- (C) or 30-min (D) delay between the end of irradiation and the beginning of the cutting of sections. In these cases, the cut and load period was 20 min.
Table II. Effects of BL on Total CWFS Peroxidase Activity, in Fractions Obtained at Varying Times after Irradiation, from Hypocotyls of 4-d-old Red Light-Grown Cucumis Seedlings

All samples were taken from CWFS according to procedures shown in Figure 1. At least four replicate experiments were performed for each treatment shown. Results are shown ± se.

<table>
<thead>
<tr>
<th>Procedure (Fig. 1)</th>
<th>BL Control 20-min harvest</th>
<th>BL 10-min harvest</th>
<th>BL Control 10-min delay</th>
<th>BL 30-min delay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase (nkat/mg protein)</td>
<td>A 185 ± 27 B 297 ± 28 C 210 ± 17</td>
<td>A 160 ± 16</td>
<td>A 246 ± 25</td>
<td>A 280 ± 30</td>
</tr>
<tr>
<td>% of dark control</td>
<td>100</td>
<td>161</td>
<td>114</td>
<td>100</td>
</tr>
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</table>

Light-grown tissue contained two diffuse anodic isoforms of peroxidase. In contrast, the solution from BL-irradiated tissue contained only one sharply focused isoform, which was more acidic than the two anodic isoforms obtained from dark-grown tissue. These differences in isozyme pattern of centrifugates obtained from control and BL-treated seedlings were observed when IEF gels were stained with either benzidine (Fig. 2) or 3-amino-9-ethyl carbazole (data not shown).

Time Course for BL-Induced Increase in Peroxidase Activity

The results of several variations in the timing of the collection of tissue for CWFS extraction are shown in Table II. When the time taken to harvest hypocotyl sections was reduced from 20 (Fig. 1A) to 10 min (Fig. 1B), the effectiveness of BL in stimulating peroxidase activity declined from 61 to 14% (Table II). This indicates a lag between the end of the BL pulse and maximal increase in peroxidase activity that probably occurs between 26 and 36 min. The duration of the BL-induced elevation in peroxidase levels in the CWFS was determined by another variation of procedure: hypocotyls were cut 10 min (Fig. 1C) or 30 min (Fig. 1D) after the end of the light pulse. These results were compared with the standard harvest technique (Fig. 1A). Increasing the time between BL treatment and centrifugation from 26 (Fig. 1A) to 36 (Fig. 1C) or 56 (Fig. 1D) min reduced the effect of light on peroxidase activity from 65% above controls at 26 min to levels not statistically different from controls at 36 and 56 min (Table II).

The relationship between the peroxidase activity of the centrifugate and the growth of intact hypocotyls following BL irradiation is summarized in Figure 3. These data show clearly that the maximum inhibition of elongation growth by BL precedes the maximum stimulatory effect of BL on peroxidase activity in the cell wall of cucumber by at least 15 min (Fig. 3). The data also show that the peroxidase level reaches its maximum after the rate of elongation has returned to the higher, control rate (Fig. 3). The effects of BL on both growth and peroxidase levels were transitory. Growth rates returned to control values within 30 to 35 min following the end of the BL treatment, and peroxidase activity returned to control levels between 40 and 45 min after the end of the BL pulse.
(Fig. 3). Note that the shortest time we achieved, using the method for obtaining the CWFS fraction described (Fig. 1), was the 25-min time point.

**DISCUSSION**

To our knowledge, the data concerning CWFS components represent the first report of the presence of NAD in the apoplast of growing tissue. Models of the redox reactions attributed to cell wall enzymes assume the presence of NAD as a cofactor (17), and the findings of this report support that assumption. Our determination of the concentration of NAD cannot be considered quantitative because of the lack of information about cell wall volume and the likelihood that some of the NAD is not removed by our procedure. Because only one method was used to detect NAD, these findings may not be definitive. Further experiments will be needed both to quantitate the NAD present in cell wall and to establish how it is transported to the cell wall space. Nevertheless, evidence of the presence of this cofactor in the apoplast of elongating tissues supports and extends current models of cell wall biochemistry.

Our conclusion that the NAD removed from the tissue sections by centrifugation is apoplastic NAD and not NAD that has leaked from the cytosol is supported by several lines of evidence. The absence of G6PDH and NADP from centrifugates and their abundance in homogenates of cucumber hypocotyls indicate little or no leakage of cytoplasmic molecules into the cell wall during centrifugation (Table I). The distribution of peroxidase isozymes between the cell wall centrifugate and tissue homogenate also indicates little or no contamination of the centrifugate by the cytoplasm. Finally, the low-speed centrifugation method has been applied to a study of apoplastic solutes in other stem tissues with results also indicating little or no cytoplasmic contamination of the centrifugate (3, 14, 18, 23, 24).

The removal of ionically bound peroxidase from the cell wall has been a commonly used technique (9, 12, 13, 19). This technique extracts more protein and peroxidase from cell walls than does the CWFS extraction procedure (Table I). The cucumber hypocotyl differs from some previously studied material (12) in that there is almost as much cytoplasmic peroxidase as there is cell wall-bound peroxidase, but this pattern has been found in other tissues as well (19). The pattern of peroxidase isozymes from the ionically bound cell wall fraction (12, 13) differs from the pattern for the CWFS as obtained here and elsewhere (3). This difference may be explained by evidence that the technique used to obtain ionically bound cell wall peroxidases is subject to cytoplasmic contamination (20).

Because the maximum BL-induced increase in peroxidase activity does not precede the BL-induced decrease in growth rate (Fig. 3), our results do not support a causal connection between total wall peroxidase activity and the decrease in growth rate. It should be noted that transient changes in peroxidase could have occurred before our first time point. However, because the peroxidase levels remain high after the growth rate has recovered to a control level, it is still unlikely that the observed BL-induced increase in peroxidase activity causes the inhibition of stem elongation induced by BL. To that extent, our results are consistent with the previous study in which rapid changes in total wall peroxidase activity did not show an inverse relationship with rapid changes in growth rate (14). In contrast, immunologically detectable activity of a specific cell wall peroxidase did change in inverse ratio with red light-induced changes in growth rate. The monoclonal antibodies used recognized a peroxidase with a single anionic isoelectric point and an unusual molecular weight (15), suggesting that this enzyme has a specialized function.

In another study, the activity of a specific anionic isofrom of peroxidase was found to decline as growth rate increased in response to end-of-day far-red light in light-grown *Sinapis* (3). The change was fast enough to account for the slower of two phases of increase in growth rate but was not as fast as the most rapid change in growth rate. This finding is similar to our findings for the BL effect on total peroxidase activity. In the previous study, no effect of BL on the activities of peroxidase isoforms was found for plants receiving 24-h treatments with red light plus or minus BL (3). Both the long-term BL treatments and the use of light-grown plants make direct comparison of these results to our findings inappropriate. At the same time, the several studies reporting changes in an anionic isofrom of peroxidase in conjunction with changes in growth rate (3, 14, 18) are consistent with a general model for peroxidase action (10) and our findings. In cucumber, BL induces a qualitative change in the anionic isoforms of peroxidase (Fig. 2). This change could affect hypocotyl elongation and might occur without an increase in total peroxidase activity. We have not yet established whether the BL-induced change in this peroxidase isoform precedes the growth response.

The BL-induced rapid transient change in cell wall peroxidase in cucumber (Fig. 3) may not cause the BL-induced inhibition of elongation, but the mechanism for the phenomenon still requires explanation. BL could increase the extractability of peroxidase from the cell wall by changing the ionic environment or physical structure of the wall. The treatment of cell walls with 50 mm CaCl₂ during extraction is probably more drastic than any internal regulatory event would be, but more subtle changes could release peroxidase not displaced by Ca²⁺. Because Ca²⁺ ions are required for the BL-induced growth response (21), and also regulate peroxidase secretion (22), BL-induced changes in cell wall Ca²⁺ could cause peroxidase secretion from the cytoplasm, leading to the delayed, transient, increase in total cell wall peroxidase activity.

**CONCLUSIONS**

The cofactor NAD is present in extracts of the apoplastic space of cucumber stems. The peroxidase obtained from the CWFS is different from that found by extraction of peroxidase ionically bound to the cell wall. BL induces a rapid transient change in total CWFS peroxidase activity that lags behind, but parallels, the BL-induced suppression in stem elongation rate. Anionic isoforms of CWFS peroxidase are specifically affected by BL, with a timing at least as fast as the overall change in CWFS peroxidase activity.
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LITERATURE CITED