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EXTRACELLULAR PEROXIDASES AS MARKERS OF STRESS?

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Many attempts have been made until now to use biochemical markers for the study of numerous physiological processes, as well as for testing the effect of several stresses on plants (10). Biochemical indicators, such as changes in enzyme activities and metabolite pools are particularly useful to provide us with an early detection of primary alterations in plants, where the naked eye does not perceive any injury at all. A major problem in using biochemical parameters as indicators of stress is that environmental factors, including light, temperature, moisture and mineral nutrition can play an important role in affecting plant responses to stress. Moreover, the responses of plants to some stresses as high salinity or water stress often are similar to those caused by other stresses, as for example, air pollution. Another important point is that stresses cause a general trouble situation which affects the whole metabolism of the plant and consequently, unspecific alterations of enzymes and membranes may occur.

Peroxidases have been frequently used as biochemical markers. In general, the stimulation of peroxidase activity in response to stress constitutes a good indication of a general increase in oxidative processes. A list of different stresses which have been found to affect the level of peroxidase activity is shown in Table 1. Peroxidases respond to these stresses by a general increase of activity, which is normally measured in crude extracts of different tissues: leaves, roots or stems. Sometimes, soluble, ionic or covalently-bound peroxidases have been
separated and the peroxidase activity measured. In other experiments, the different molecular isoforms have been separated showing, in a few cases, the appearance of new isoperoxidases due to stress (7).

Table 1  Different stresses which affect the level of peroxidase activity in plant tissues

<table>
<thead>
<tr>
<th>Stresses</th>
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</thead>
<tbody>
<tr>
<td>Cold, freezing</td>
</tr>
<tr>
<td>Flooding, moisture</td>
</tr>
<tr>
<td>Hypogravity</td>
</tr>
<tr>
<td>Hypoxia</td>
</tr>
<tr>
<td>Infection (viruses, bacteria, fungi)</td>
</tr>
<tr>
<td>Ion status (deficiency, toxicity)</td>
</tr>
<tr>
<td>Mechanical injury (wounding, irritation)</td>
</tr>
<tr>
<td>Pollution: air pollutants (SO₂, NOₓ, O₃, PAN, HF, HCl...)</td>
</tr>
<tr>
<td>: heavy metals (Cd, Pb, Hg, Ni, Zn, Cr...)</td>
</tr>
<tr>
<td>Radiation (UV, X, γ)</td>
</tr>
<tr>
<td>Salt stress</td>
</tr>
<tr>
<td>Water stress, drought</td>
</tr>
</tbody>
</table>

In spite of the general stimulation of peroxidase activity in response to different stresses, several complications exist in establishing such biochemical diagnostic test as an indicator of stress. The principal problem is that peroxidase activity is affected not only by environmental factors but also by the internal physiological state of the plant (10). It is well known that peroxidase activity is modified by several physiological processes as senescence, root formation, flower initiation, abscission, etc. (10). Another problem is that peroxidase activity varies largely and inconsistently within the day and between individual plants. This variability would depend on genetic characters. Other additional factors are that peroxidases lack specificity for a particular stress and sometimes they are not connected with damage or symptoms. Some of these factors could reduce the diagnostic value of peroxidase activity and show how difficult the interpretation of results can be if they are not taken into account.

In addition to that, there are other points that are never discussed in works concerning the use of peroxidase as indicator of stress. As pointed out before, most experiments are performed by measuring total peroxidase activity in more or less crude extracts. By this way, a source of error can be introduced by the presence of many substances (phenolics, organic acids, ions) which could act as positive or negative effectors of the enzyme (4, 5, 14). These compounds are probably separated from peroxidases in the intact cells as a result of different
compartmentation. When cells are broken, these compounds are mixed with enzymes and interact with them during the assay, modifying the capacity of peroxidases to oxidize the electron donors. Consequently, the physiological meaning of measurements made from such extracts is subject to discussion.

Some of these problems can be solved by using an alternative technique, i.e., the vacuum infiltration of a liquid into the plant tissue, followed by the recovery of the fluid by centrifugation. By this way, the peroxidase activity present in the extracellular space may be quantified. Indeed, some controls must be done to be sure that cell integrity is maintained (enzyme markers for cytoplasm, chloroplast or vacuole contamination). This method allows us to modify the ionic strength of the infiltration medium in such a way as to withdraw either free or ionically-bound peroxidases. Peroxidase activity measured in extracellular fluid may also supply some information on the rate of release of peroxidases out of the protoplast, which has been shown to be stimulated after stress (6).

Table 2. Ratio of extracellular to total peroxidase activities from control and ozone-treated plant leaves at different times after a 2-h ozone exposure. Peroxidase activity was assayed with three different electron donors.

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>ozonized</th>
<th>0h</th>
<th>3h</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guaiacol</td>
<td>+</td>
<td>0.086*</td>
<td>0.084</td>
<td>0.087</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.047</td>
<td>0.048</td>
<td>0.048</td>
<td>0.049</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>+</td>
<td>0.054</td>
<td>0.092</td>
<td>0.058</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.011</td>
<td>0.012</td>
<td>0.011</td>
<td>0.011</td>
</tr>
<tr>
<td>Syringaldazine</td>
<td>+</td>
<td>0.011</td>
<td>0.013</td>
<td>0.016</td>
<td>0.017</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.011</td>
<td>0.012</td>
<td>0.011</td>
<td>0.011</td>
</tr>
</tbody>
</table>

*Standard deviations were less than 10% of the mean for control and ozone-treated plants

Extracellular peroxidases, which include enzymes present in the intercellular spaces and enzymes ionically-bound to cell walls, are known to be involved in the polymerization of lignin precursors (12), suberization (1), and the cross-linking of proteins or other molecules with cell wall material (20). Peroxidases may also be useful by hardening cell membranes by the formation of bridges between tyrosine residues of membrane proteins (8). Such a reaction could allow plants to reduce their cellular
permeability. An increased peroxidase activity may also be linked to a decreased rate of growth (10).

As an example of this approach some results concerning extracellular peroxidases of *Sedum album* leaves exposed to oxidant stress will be presented here. It was previously shown that extracellular peroxidases are more sensitive to oxidant stress than those from cell material (6). Consequently, the ratio of extracellular to total peroxidase activities will be enhanced in stressed plants. This ratio constitutes an internal parameter of the plant and could be useful as a marker of stress. Table 2 shows the ratio values calculated from peroxidase activities measured in *Sedum album* leaves at different times after one exposure to 0.4 ppm ozone for 2 h. Peroxidase activity was assayed with three different electron donors: guaiacol, ascorbate and syringaldazine. When peroxidase activity is measured with ascorbate as electron donor, the ratio increases rapidly (as soon as 3h after ozone exposure), this increase being already perceptible just at the end of the exposure. When guaiacol is the electron donor, the corresponding ratio is hardly modified just after ozone exposure but it increases 2.2 times in ozone treated plants 48h later. In the case of syringaldazine, the ratio exhibits a progressive increase after ozone exposure to reach maximum values 24 and 48h later.

Figure 1a shows the isoperoxidase patterns of intercellular fluid and cell material of *Sedum album* leaves obtained by agarose gel isoelectric focusing 3 and 24h after ozone exposure. Peroxidase activity was revealed by using benzidine as electron donor. The main bands are found in the acidic part of the pH gradient and near pH 11 . Exposure to ozone does not induce new isozyme bands and no great differences between control and ozone treated leaves are observed. A linear relationship is obtained between the amount of protein in the agarose gel and the colour intensity developed with benzidine as shown by the relative values of optical density obtained by photodensitometry (Fig. 1b). Ozone exposure rapidly stimulates the extracellular basic peroxidase activity (as soon as 3h after the end of exposure), whereas extracellular acidic peroxidases reach maximum activity 24h later.

The above results and other obtained before (3), can be summarized in the sequence of reactions shown in Figure 2. Different stresses, in our case ozone, could provoke the formation of several kinds of free radicals, which may initiate lipid peroxidation (19). The degradation of lipoprotein cell membranes by free radical reactions may induce changes in ionic status and fluxes at the plasmalemma level (18). In our own experiments with *Sedum album* leaves, extracellular K+ increased by 15% after ozone exposure as compared to untreated plants. This release of potassium could result in a modified distribution of
Fig. 1a. Zymogram patterns of *S. album* peroxidase isozymes of control and ozone-treated plants, 3h and 24h after a 2-h ozone exposure. Peroxidase activity was revealed with benzidine. Anode at the top. IF, intracellular fluid (4 µg protein/sample); CM, cell material (20 µg protein/sample). Relative absorption values of peroxidase activity were obtained with a Photointegrator Vernon PHIS. Hundred units correspond to the absorption of the cell material peroxidase activity in control plants (acidic + basic peroxidases).

Fig. 1b. Quantitative estimation of peroxidase activity by staining with benzidine. In the lower panel, zymogram patterns of acidic peroxidases containing 5 to 20 µg protein from the same sample. Upper panel shows the linear relationship between the areas of integration peaks and the amounts of protein used for each sample.
Ca\(^{2+}\) which, in turn, can play a crucial role in the response of peroxidase to ozone. Indeed, an increase in the cytoplasmic Ca\(^{2+}\) concentration is required for peroxidase secretion (6, 16) and newly secreted peroxidases could be activated by the incorporation of Ca\(^{2+}\) once they are in contact with extracellular spaces, rich in Ca\(^{2+}\). Thus, the enhancement of extracellular peroxidase activity after an ozone treatment would be not only a consequence of the activation of peroxidases themselves by calcium ions but also a consequence of newly synthesized peroxidase molecules arriving at the extracellular spaces of ozone treated leaves (3).

Fig. 2. Possible pathway of peroxidase reactions in response to oxidant stress (ozone). See text for further details.

Ozone exposure also provokes a rapid stimulation of ethylene production which follows the increase in basic peroxidase activity (3). This sequence of events, which has also been observed in response to mechanical stimulus in Bryonia dioica (2), raises the question of the possible relationship between ethylene and peroxidases (9). It has been suggested that the enzyme transforming ACC to ethylene could present some characteristics of peroxidases (15), and a good correlation between the activity of membrane-bound peroxidases and ethylene production has been observed (2,13). Peroxidases would directly control ethylene production by means of ACC oxidation, and ethylene in turn, could regulate acidic peroxidases thereby controlling also the lignification process (17).
The stimulation of peroxidase activity at the exterior of the cells can have multiple effects at the physiological level. The control of auxin by basic peroxidases would reduce growth (see 9 for references). They could also play a detoxifying role as oxidant scavengers using ascorbic acid as electron donor (a simultaneous release of basic peroxidase and ascorbic acid into free space is observed in response to ozone, 3). A low oxidant level maintained in the extracellular environment could allow the cells to withstand pathogens more effectively. The enhanced acidic peroxidase activity would accelerate the lignification processes (11, 17) increasing the resistance against infection or other stresses.

Several points have to be taken into account if we want to ameliorate the "peroxidase test" as a marker of stress. First of all, the compartmentation of the cell. By avoiding the disruption of the cell integrity, we can detect the physiological activation of peroxidases in response to stress. For example, in some pollution studies, total peroxidase activity is not modified by low pollutant concentrations, whereas the extracellular activity is highly stimulated. Secondly, the specificity of free spaces as a preferential meeting site between some isoenzymes and their natural specific electron donors and acceptors needs to be substantiated. Finally, a better comprehension of the activation mechanism of peroxidases in response to stress is required.

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