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SUPEROXIDE DISMUTASE IN WOUNDED ETIOLATED PEA SEEDLINGS

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Key Word Index—*Pisum sativum*; Leguminosae; pea; wound response; superoxide dismutase; SOD; catalase; peroxidase.

Abstract—The activities of three related enzymes, superoxide dismutase, peroxidase and catalase, were followed in decapitated (wounded) etiolated pea stem tissue. Of the three enzyme activities, only that of superoxide dismutase showed a significant loss and recovery pattern within 1 hr. The change in enzymatic activity appears to result from protein loss and recovery.

INTRODUCTION

Under normal conditions both photosynthetic and mitochondrial processes have the potential for the production of adventitious free superoxide radicals [1]. Superoxide dismutase enzymes as well as reduced glutathione, ascorbic acid and alpha-tocopherol present in the cell provide protection against these radicals and consequently protect against the more hazardous hydroxyl radical and singlet oxygen molecules which can arise via superoxide reactions [2]. The wounding of plant tissue, due to the disruption of cell structures, increases the possibility of superoxide generation by the release of compounds such as ferridoxins, diphenols, pteridines and reduced flavins which are known to autoxidize in the presence of oxygen to produce superoxide radicals [1, 3]. Enzymes such as galactose oxidase, xanthine oxidase and certain peroxidases are also capable of producing these radicals as part of their catalytic function [4–6].

The superoxide radical has been shown to be an antifungal agent. Doke has observed enzymatic production of these radicals in response to certain hyphal wall components in potato tuber protoplast as well as in other systems including pea [7]. The build-up of superoxide radicals was shown to be suppressed by addition of exogenous superoxide dismutase.

The question of cellular control of these radicals under wound conditions led us to investigate the effects of wounding on the activity of the plant cell's protective system, the superoxidase dismutases, catalases and peroxidases.

The etiolated pea seedling stem tissue selected for this study is known to contain two copper zinc bearing isoenzymes of superoxide dismutase, CuZnSOD I and CuZnSOD II, the latter of which is exclusive to the chloroplast [8]. A third isoenzyme contains manganese. Early reports had placed the MnSOD isoenzyme in the mitochondria [8–10]; more recently, it has been reported to be located exclusively in the peroxisomes [11]. The manganese-containing enzyme can be distinguished from the other two by its resistance to cyanide inactivation [12].

The hydrogen peroxide generated in the dismutation reaction can be acted on by either the peroxidase or the catalase present in the cell. Pea tissue has been shown to contain multiple forms of peroxidase, two of which occur at cut stem surfaces. Total peroxidase activity has been reported to increase over at least a 30 hr time period following wounding [13].

RESULTS AND DISCUSSION

The evaluation of SOD activity was done on crude stem tissue extracts using a spectrophotometric assay [12]. In spite of the potential interference by compounds such as glutathione, ascorbate, metal ions and photosynthetic electron-transfer compounds which may react with superoxide radicals, this type of assay should indicate the ability of the stem tissue at the wound site to deal with superoxide radicals. To avoid the potential complications of handling the seedlings under varied lighting conditions, the experiments were performed with etiolated tissue under safe lights.

Figure 1 shows the SOD activity in the stem tissue measured in solution vs. the time post wounding. The

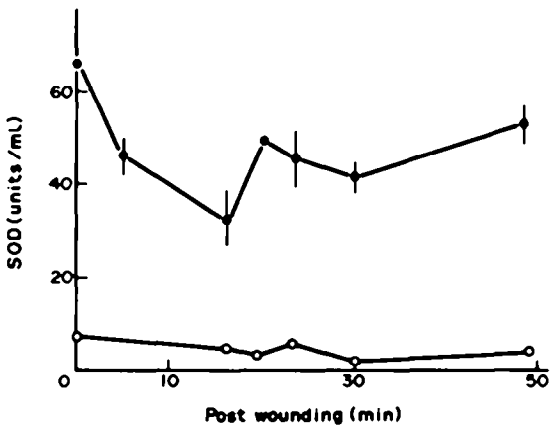

Fig. 1. SOD activity, in solution assay, of etiolated pea seedling stem vs. time post wounding. (●) Total SOD, (○) MnSOD. The results were compiled from four separate experiments and represent 32 stem sections per time point. The vertical bars represent ± s.d.

changes can be viewed as occurring in two phases, the first being a rapid decline in SOD activity returning to *ca* 80% of the initial level within 1 hr of wounding. Assay in the presence of cyanide indicates that under these conditions the Mn-containing isoenzyme contributes no more than 11% of the total dismutase activity, and undergoes relatively insignificant changes by comparison to the overall changes in SOD activity.

Catalase and peroxidase activity, which enzymatically could couple to the products of the superoxide dismutation reaction, do not follow this biphase pattern. In both cases there were declines in activity, catalase to 67% of the original, peroxidase to 56% of the original over a 100 min period following decapitation. Ockerse and Mumford [13] have shown that in excised pea stem segments (Progress No. 9) two of the nine peroxidases present are produced in response to wounding; however, this increase in active peroxidases did not occur until 3 hr post wounding and then continued for the remaining 3 hr of their experimental period. This is consistent with our own observation of a decline in peroxidase activity during the first 100 min after wounding.

Of the three enzyme activities, only that of SOD responded in a quick and dramatic way to wounding. The relative activity of each of the three SOD isoenzymes was determined using gel electrophoresis. Figure 2 shows the negative absorbance peak heights from one of the four sets of data used in Fig. 1. Using this technique the percentage of MnSOD present in the stem homogenate is 30%, a value three times that measured using the solution assay. The difference may reflect an inhibitory effect of the cyanide, found in the solution assay, on MnSOD. CuZnSOD I and CuZnSOD II, as designated by Duke and Salin [8], respectively, make up 40 and 30% of the total SOD activity. The plot shows a fairly uniform pattern for all three isoenzymes, with the minimum activity occurring somewhat later than that in the solution assay. At 30 min, both CuZnSODs are reduced to *ca* 20% and the MnSOD is reduced to approximately 12% of the 5 min value. At 50 min the recovery in activity for CuZnSOD I and II is 54 and 77%, respectively, and that of MnSOD is at 23% of the 5 min level. Data for a single experiment assayed by both procedures show some differences; however, both techniques indicate a definite pattern of rapid loss and subsequent recovery of SOD activity in the decapitated stem tissue. The initial loss of activity may be due to protein degradation or to some form of enzyme inhibition. The recovery phase may be the result of protein synthesis, reversal of inhibition or activation of precursor forms of the SOD proteins.

Lowry assays for total soluble protein in the tissue homogenates show a pattern similar to that for SOD but with longer recovery times. Figure 3 illustrates the decrease in protein content for the three SOD isoenzymes as well as a slight recovery, as measured on Coomassie blue stained gels.

In an attempt to isolate the initial phase from the recovery phase, tissue homogenates of unaged pea stem sections, prepared in a manner analogous to that used for the timed experiments, were incubated at 25°. The reactions were stopped at various time intervals by freezing at −20°. Gel assay showed a decline in both protein and activity associated with the SOD bands. The experiment showed only a decline phase, indicating the susceptibility of the SOD proteins to endogenous proteases. It also indicates that the recovery phase must require components not present in the crude tissue homogenate. The recovery phase, based on both the activity and protein-stained gels, appears to be dependent upon the generation

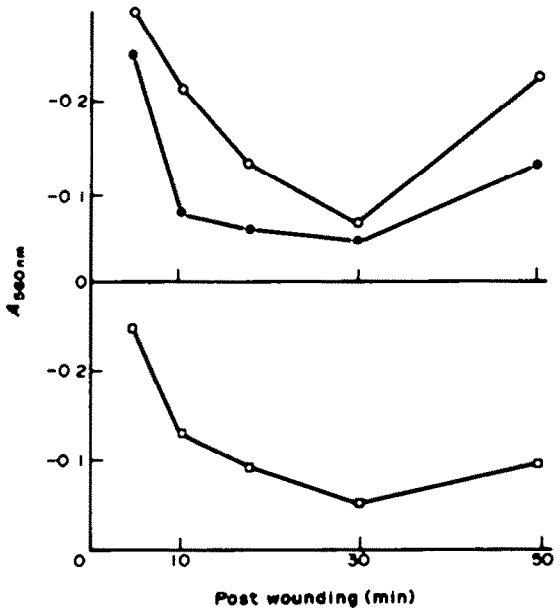

Fig. 2. Negative absorbance peak height of SOD isoenzymes electrophoresed on Davis gels and stained for SOD activity vs. time post wounding. (○) CuZnSOD II; (●) CuZnSOD I; (□) MnSOD.

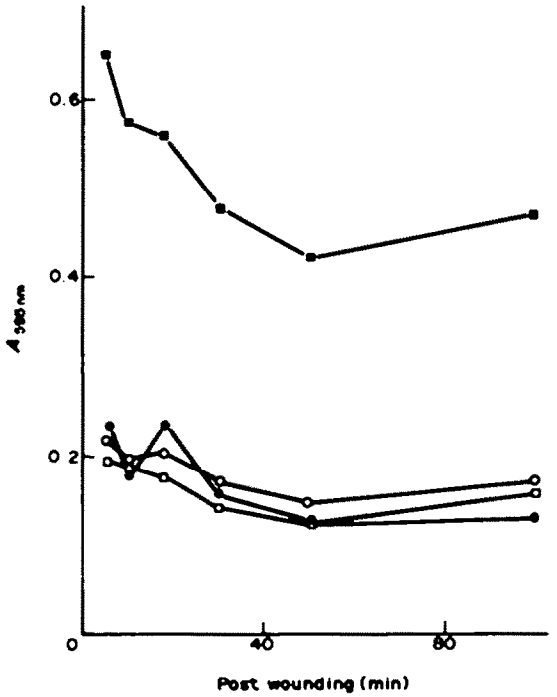

Fig. 3. Absorbance peak heights of Coomassie blue stained SOD isoenzymes separated on Davis gels vs. time post wounding. (■) Total SOD, (○) CuZnSOD II, (●) CuZnSOD I, (□) MnSOD.

of active SOD proteins. Experiments in which either actinomycin or cycloheximide solutions were applied to the decapitated stem surface gave inconclusive results as to the level at which synthesis may be occurring. This may have been due to difficulties in uptake of the inhibitors by the tissue within the short time-frame of the experiment.

These observations add to the growing understanding of plant wound response. Work done on the enzymatic generation of superoxide anions in response to certain hyphal wall components [7] suggests the utility of the superoxide radical as an antifungal agent. The rapid decline in SOD in response to wounding may be a preparatory action for such a defence system, allowing the formation of superoxide radicals at the wound site by reducing the levels of endogenous SOD which would otherwise suppress the enzymatic production of superoxide stimulated by the hyphal wall component.

EXPERIMENTAL

Pea seed (*Pisum sativum* L., cv. Alaska) was disinfected and germinated at 27° in the dark under asceptic conditions on sterile filter paper [14]. When the epicotyls reached 4 mm, the seedlings were decapitated and planted in sterile media (Gibco B-5 media without hormones, 0.6% agar). The cut stem surfaces were treated with 4 μl 3% EtOH in sterile H_2O. This was done as a control for other expts using this system. Stems which had not been treated with EtOH showed similar results. Tissue was sampled vs. time using eight seedlings per time point and excising approximately 2 mm of stem tissue below the decapitation point. All tissue manipulations were done under red safe lights.

Stem sections were frozen at −20°. Frozen stem samples (eight stem sections per sample) were homogenized in 0.75 ml 0.05 M phosphate buffer (pH 7.8) using a Tissumizer homogenizer. After refreezing and thawing, the samples were centrifuged at 12000 rpm in an Eppendorf microfuge for 2 min. The resulting supernate was used for analysis.

Total SOD activity was measured in soln by an inhibition assay, as described by Beauchamp and Fridovich [12], using xanthine–xanthine oxidase to generate superoxide, and nitroblue tetrazolium as the superoxide detector. The procedure was modified to use 1 mM diethylaminetriaminepentaacetic acid rather than EDTA [15]. MnSOD was determined after preincubation of the sample with 5 mM cyanide for 30 min prior to assay.

The determination of total SOD activity in a sample required 6 separate assays at varied sample concns. A computer calculation, which plotted the ratio of the changes in absorbance per min for the blank to the change in A per min for the sample reaction vs. the sample vol. was used to calculate the 50% inhibition vol. This was designated to contain 1 unit of SOD activity when the blank values were in the range of 0.3–0.4 change in A per min. MnSOD was determined by the same procedure using samples which were pre-incubated with cyanide. The difference between the total SOD and MnSOD activities was assumed to be the activity due to the two CuZnSOD isoenzymes.

In a gel assay, isoenzymes of SOD were separated electrophoretically on 10% Davis gels, pH 8.9 [16]. The gels were stained as described by Kaars and Kosman [17] using nitroblue tetrazolium and riboflavin. The assay produces clear bands of enzyme activity on a blue background. A Beckman DU-8 equipped as a densitometer was used to scan the gels at 560 nm. The negative absorbance peak height was used as a measure of activity. The correlation between negative absorbance peak height and SOD activity, as determined in a soln spectrophotometric assay also using nitroblue tetrazolium, is linear.

Total soluble protein was determined by the Lowry method [18]. Relative protein concns of the SOD isoenzymes were determined from densitometric scans of Coomassie blue stained Davis gels. The appropriate bands were identified by comparison of R_f factors with duplicate gels stained for SOD. The positive absorbance peak height at 595 nm was used as a measure of protein concn.

Peroxidase assays were performed according to the procedure of Ockerse and Mumford using guiacol and H_2O_2 as substrates [13]. Catalase assays were performed with an oxygen monitor (Yellow Springs Instrument Co., Model 53) equipped with a Clark electrode. Aliquots of tissue homogenate were used to initiate the catalase reaction with 15 mM H_2O_2 in 50 mM, pH 7.0 K-Pi buffer. Reactions were run at 25°.

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REFERENCES

1. Fridovich, I. (1978) *Science* **201**, 875.
2. Halliwell, B. (1982) in *Superoxide Dismutase* (Oberly, L. W., ed.) Vol. 1, p. 89. CRC Press, Boca Raton.
3. Mishra, H. P. and Fridovich, I. (1971) *J. Biol. Chem.* **246**, 6886.
4. Hamilton, G. A., Adolf, P. K., deJersey, J., DuBois, G. C., Dyrkacz, G. R. and Libby, R. D. (1978) *J. Am. Chem. Soc.* **100**, 1899.
5. McCord, J. M. and Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049.
6. Halliwell, B. (1978) *Planta (Berlin)* **140**, 81.
7. Doke, N. (1983) *Physiol. Plant Pathol.* **23**, 359.
8. Duke, M. V. and Salin, M. L. (1983) *Phytochemistry* **22**, 2369.
9. Foster, J. G. and Edwards, G. E. (1980) *Plant Cell Physiol.* **21**, 895.
10. Sevilla, F., Lopez-Gorge, J. and del Rio, L. A. (1982) *Plant Physiol.* **70**, 1321.
11. del Rio, L. A., Lion, D. S., Olah, I., Glick, B. and Salin, M. L. (1983) *Planta* **158**, 216.
12. Beauchamp, C. and Fridovich, I. (1971) *Analyt. Biochem.* **44**, 276.
13. Ockerse, R. and Mumford, L. M. (1973) *Can. J. Botany* **51**, 2237.
14. Kurkdjian, A., Manigualt, P. and Beardsley, R. (1974) *Am. Biol. Teacher* **26**, 13.
15. Buettner, G. R., Oberly, L. W. and Chan Leuthauser, S. W. H. (1978) *Photochem. Photobiol.* **38**, 693.
16. Gabriel, O. (1971) in *Methods in Enzymology* (Jakoby, W. B., ed.) Vol. XXII, p. 565. Academic Press, New York.
17. Kaars, J. and Kosman, D. (1983) *J. Chem. Educ.* **60**, 1082.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.